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# Targeting the adenosine system to limit ischemia-reperfusion injury

Translational research in humans

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# Targeting the adenosine system to limit ischemia-reperfusion injury

Translational research in humans

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# 1

General introduction  
and outline of the thesis





## General introduction

### Background

Coronary heart disease (CHD) is the leading cause of death and disability worldwide. According to the World Health Organisation, an estimated 7.4 million deaths worldwide (13.2% of all deaths) result from CHD each year.<sup>1</sup> Despite major advances in prevention and treatment, morbidity and mortality in patients with an acute myocardial infarction remain significant. In the setting of a myocardial infarction, acute occlusion of a coronary artery induces focal myocardial ischemia. Establishing early myocardial reperfusion is the most effective strategy to reduce myocardial injury and limit infarct size to improve clinical outcome.<sup>2</sup> Restoration of blood flow to the ischemic myocardium itself however, can also contribute to myocardial injury. This phenomenon is called 'lethal reperfusion injury'.<sup>3</sup> During early reperfusion various intracellular and extracellular events occur, including increased oxidative stress, pH changes, calcium overload, an acute inflammatory response and metabolic changes which cause tissue injury themselves and contribute to final myocardial infarct size.<sup>3</sup> Studies in animal models of myocardial infarction suggest that up to 50% of the final infarct size is accounted for by reperfusion injury.<sup>3</sup> Therefore, additional strategies that could limit ischemia and reperfusion (IR) injury might have a significant impact on the prognosis following myocardial infarction. Both pharmacological and non-pharmacological interventions have been developed with the aim to prevent myocardial IR-injury.

### Pathophysiology of myocardial ischemia reperfusion injury

#### Ischemia

During myocardial ischemia, the arterial blood flow cannot provide enough oxygen to meet the energy demands of the tissue and therefore to maintain tissue function.<sup>4</sup> Within seconds after occlusion of a coronary artery (and in absence of collateral interarterial connections), anaerobic glycolysis becomes the dominant energy-generating pathway. This anaerobic glycolysis will lower cell pH.<sup>4</sup> To compensate for this accumulation of hydrogen ions, the  $\text{Na}^+/\text{H}^+$  exchanger removes the excess hydrogen ions in exchange for sodium ions, which produces a large influx of sodium ions. Ischemia also depletes cellular ATP which inactivates ATPases, reduces active  $\text{Ca}^{2+}$  efflux and limits reuptake of calcium by the endoplasmic reticulum, thereby producing calcium overload in the cell.<sup>5</sup> In the heart these cumulative metabolic

changes will ultimately lead to damaged myofibrils, hypercontracture and contracture band necrosis. The extent of tissue injury depends on the magnitude and duration of the ischemic stimulus.<sup>6</sup>

### Reperfusion injury

Although reperfusion is essential to prevent complete necrosis of the myocardial cells by restoring the delivery of oxygen and substrates required for aerobic ATP generation and normalization of extracellular pH by washing out accumulated  $H^+$ , reperfusion itself also has detrimental consequences<sup>3</sup>. As stated before, the process of reperfusion triggers various events, including an increase in reactive oxygen species (ROS), decrease in intracellular pH, calcium overload, an acute inflammatory response and metabolic changes, which ultimately cause endothelial dysfunction and tissue injury themselves and contribute to the final myocardial infarct size.<sup>3</sup> Many of these factors promote the opening of the mitochondrial permeability transition pore (mPTP), a critical determinant of cell death in the setting of acute IR-injury. Opening of this channel induces a collapse of the mitochondrial membrane potential and uncouples oxidative phosphorylation, resulting in ATP depletion and cell death.<sup>7</sup> In clinical practice, myocardial reperfusion injury has a number of detrimental clinical consequences. First, the sudden reperfusion of ischemic myocardium may be accompanied by ventricular arrhythmias. Second, due to oxidative stress and intracellular calcium overload reversible post-ischemic contractile dysfunction occurs which has been termed myocardial stunning. Third, myocardial reperfusion injury may lead to endothelial damage with impaired vasodilation and endothelial cell swelling which may impede adequate restoration of blood flow. At last, reperfusion-induced death of cardiomyocytes may occur, a phenomenon that is referred to as 'lethal reperfusion injury'.<sup>8</sup>

### Prevention of ischemia reperfusion injury

With the beneficial effects of early reperfusion well established, but offset by reperfusion injury, efforts have been put forward to reduce reperfusion injury and improve outcome. Of all myocardial infarction patients, 15% are hospitalized within the first year and of these patients one-year mortality is up to 50%.<sup>9</sup> If further improvement in survival and quality of life is desired, strategies to limit not only ischemia, but also reperfusion injury should be developed. Currently, percutaneous interventions (PCI) and coronary artery bypass grafting (CABG) are the most important techniques to restore myocardial perfusion and therapeutic strategies to limit reperfusion injury in clinical practice should be related to these therapeutic strategies.

### (Pharmacological) conditioning

Already in 1986, Murry et al. reported that myocardial infarct size could be reduced by brief intermittent periods of myocardial ischemia and reperfusion preceding the lethal ischemic insult, a phenomenon which has been termed '*ischemic preconditioning*'.<sup>10</sup> Robust infarct-size limitation by ischemic preconditioning has been confirmed repeatedly in various animal species and in different organs (e.g. kidney, brain).<sup>11-13</sup> Two different phases of preconditioning can be distinguished: an 'early phase' which induces immediate protection but only for the first 2 hours after the preconditioning stimulus and a 'delayed form', which reappears after approximately 24 hours after the preconditioning stimulus and lasts longer but provides weaker protection.<sup>14</sup>

Despite the attractive properties of ischemic preconditioning, application in the real life setting is limited as ischemic events, e.g. acute myocardial infarction, occur randomly and unexpected. To overcome these limitations, other conditioning strategies were developed. In 2003 Zhou et al. were the first to describe the concept of '*ischemic postconditioning*'.<sup>15</sup> Where preconditioning is triggered by brief episodes of ischemia and reperfusion performed just before a prolonged coronary artery occlusion, postconditioning is induced by a comparable sequence of reversible ischemia and reperfusion but is applied just after the prolonged ischemic event.<sup>16</sup> In dogs a single cycle of 5 minutes left anterior descending artery occlusion and 10 minutes of reperfusion reduced myocardial infarct size induced by a subsequent 60 minute LAD occlusion. Several clinical studies already suggest that postconditioning after coronary angioplasty protects the human heart during acute myocardial infarction.<sup>16</sup> The observation that postconditioning limits final infarct size is the most direct illustration that reperfusion injury exists and importantly contributes to infarct size.

Interestingly, the heart is protected against IR-injury not only by brief preceding preconditioning episodes of the heart itself, but also when this preconditioning stimulus is applied to other organs than the heart, called *remote ischemic preconditioning (RIPC)*. In RIPC brief, reversible episodes of ischemia and reperfusion applied to one vascular bed, tissue or organ induce global protection, rendering remote tissues and organs resistant to subsequent ischemia and reperfusion.<sup>17</sup> In the past decades, the concept of RIPC has been successfully explored, showing that protection against IR-injury can be transferred between various organs and tissues in different species; e.g. brief occlusion of the renal artery protects the heart against a subsequent ischemic insult, or brief occlusion of the limb protects the kidney against a subsequent ischemic insult.<sup>17</sup>

How does ischemic conditioning protect the heart? Up to now, thousands of studies have been published which report more than 100 different signalling molecules and mechanisms of conditioning. Briefly, during the preconditioning ischemia, several substances are released that 'trigger' protection of myocardial cells. These triggers include adenosine, bradykinin, reactive oxygen species (ROS), opioids and nitric oxide. Binding of these triggers to receptors activates several intracellular signalling pathways, such as the reperfusion injury salvage kinase (RISK) pathway, endothelial NOS/protein kinase G (PKG) pathway and the survival activating factor enhancement (SAFE) pathway.<sup>18</sup> Finally, the state of the mPTP is crucial for cell survival. Opening of the mPTP at reperfusion leads to dissipation of the inner mitochondrial membrane potential and this ultimately results in cellular desintegration.<sup>18</sup> Most prosurvival pathway ultimately converge on the mPTP and prevent the mPTP from opening at reperfusion and therefore salvage these cells.

### **Pharmacological preconditioning: targeting the adenosine pathway**

Now that we know the intracellular signalling pathways that are activated by ischemic preconditioning, it is tempting to try to mimic the cardioprotective effect by drugs that activate these pathways. In preclinical studies, both pharmacological activation of the RISK pathway and pharmacological prevention of the mPTP opening reduces myocardial IR-injury.<sup>3</sup> Drugs that effectively reduce myocardial infarct size in animal models include statins, erythropoietin, atrial natriuretic peptide, cyclosporine, adenosine, dipyridamole, and metformin.<sup>3,19</sup> The endogenous nucleoside adenosine is one of the main ligands that trigger activation of the prosurvival pathways during ischemic preconditioning.<sup>20</sup> Interestingly, intravenous administration of exogenous adenosine also reduced infarct size by 75% in animals.<sup>21</sup> Later, these protective effects of adenosine were confirmed in human atrial tissue in vitro and in human in vivo experimental models.<sup>22,23</sup> In the Acute Myocardial Infarction Study of ADenosine (AMISTAD) I and II trials, adenosine was used as an adjunct to thrombolytic treatment or primary angioplasty in patients with STEMI.<sup>24, 25</sup> The results of the AMISTAD I were encouraging in those patients who had anterior myocardial infarction and in those who were treated with higher doses of adenosine. The larger AMISTAD II trial, in which adenosine was administered intravenously for 3 hours, 15 minutes before the start of thrombolytic therapy or coronary intervention, failed to demonstrate an effect on morbidity and mortality.<sup>25</sup> In a retrospective analysis of the AMISTAD II data, Kloner et al. did find a significant reduction of infarct size in a subgroup of patients who were treated early after the onset of symptoms.<sup>26</sup>

So, the adenosine pathways is an attractive pharmacological target to mimic ischemic conditioning. The administration of adenosine, however has several drawbacks: 1) an extremely short plasma half-life of less than 1 second, and 2) a strong metabolic

barrier function of the endothelium for adenosine, prevent effective availability of adenosine in the interstitial compartment.<sup>27</sup>

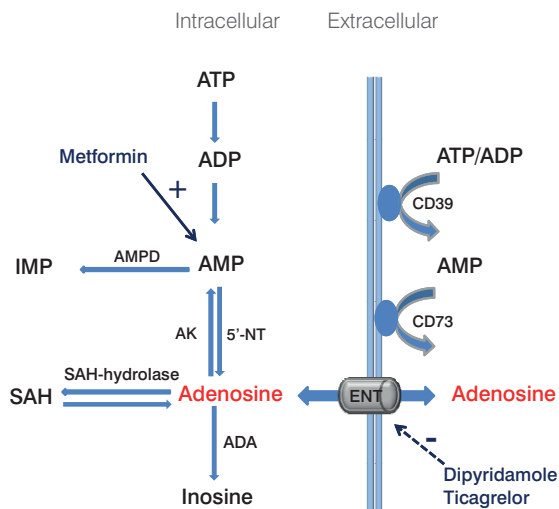
Furthermore, potential side effects of intravenous infusion of adenosine are the occurrence of cardiac arrhythmias, and hypotension.<sup>28-30</sup> Thus, although the key role of endogenous adenosine in the protective mechanisms of ischemic conditioning is indisputable, the intravascular injection of adenosine is not the most appealing method to acquire the desired cardioprotection.

To circumvent the abovementioned limitations of administering adenosine, drugs have been developed to increase the endogenous adenosine concentration. To understand how pharmacological compounds can increase the endogenous adenosine concentration, we will briefly describe the metabolism of adenosine. The physiological effects of endogenous adenosine depend on the extracellular adenosine concentration, which is a net result of intracellular and extracellular formation and degradation of adenosine (see figure 1). For this reason, several pharmacological methods to increase the extracellular concentration of endogenous adenosine have been explored.

### *The adenosine metabolism*

Under ischemic circumstances, changes in the metabolism of adenosine (see figure 1) result in a rapid increase of the extracellular adenosine concentration.<sup>31,32</sup> Adenosine is formed intracellularly mainly by cytosolic 5'-nucleotidase, which dephosphorylates AMP. A second, but less important pathway, is hydrolysis of S-adenosylhomocysteine. AMP is competitively converted by the enzyme AMP deaminase (AMPD) into inosine monophosphate (IMP).<sup>33</sup> In cardiomyocytes, under well-oxygenated conditions, AMP is mainly converted to IMP, whereas in ischemic or hypoxemic situations, conversion to adenosine is predominant.<sup>33-35</sup> Extracellular formation of adenosine is mediated by dephosphorylation of AMP via membrane-bound ecto-5'-nucleotidase (CD73).<sup>33,36</sup> Degradation of adenosine occurs mainly in the intracellular compartment, where adenosine is rephosphorylated to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase.<sup>33</sup>

During well-oxygenated conditions approximately 90% of adenosine is produced intracellularly.<sup>37</sup> The cytosolic adenosine concentration remains very low due to the effective intracellular rephosphorylation of adenosine to 5'-AMP by adenosine kinase and this in turn leads to a transmembrane concentration gradient driving extracellular adenosine into the cytosol under normal physiological conditions.<sup>37</sup> Adenosine is a relatively hydrophilic molecule and therefore transport of adenosine across the cellular membrane is facilitated by a concentration gradient driven equilibrative



**Figure 1** Schematic overview of the metabolism of adenosine and the effect of pharmacological agents studied in this thesis.

ADA, adenosine deaminase; ADP, adenosine diphosphate; AK, adenosine kinase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CD 73, ecto-5'-nucleotidase; ENT, equilibrative nucleoside transporter; CD 39, ecto-nucleoside 5'-triphosphate diphosphohydrolase; IMP, inosine monophosphate, SAH, S-adenosylhomocysteine.

nucleoside transporter (ENT).<sup>38</sup> Currently, four different subtypes of ENT are described (ENT 1-4), ENT1 and 2 being the most important for adenosine transport in humans<sup>39</sup>

During ischemic or hypoxemic conditions, the extracellular adenosine concentration increases due to several adaptation in the metabolism: hypoxia upregulates ecto-5'-nucleotidase (CD73) and ecto-nucleoside 5'-triphosphate diphosphohydrolase (CD39) which will increase extracellular adenosine formation.<sup>40,41</sup> Second, a reduction in intracellular degradation due to hypoxic inhibition of adenosine kinase will reduce the transmembrane concentration gradient which will diminish the influx of extracellular adenosine.<sup>42</sup> Finally, a reduction in the transport capacity of ENT's may reduce cellular uptake of adenosine and therefore increase extracellular adenosine concentration.<sup>43,44</sup>

### *Drugs that influence adenosine signalling*

This thesis focuses on three drugs that potentially target the adenosine pathway: metformin, dipyridamole and ticagrelor.

#### **Metformin**

Several large observational studies in patients with type 2 diabetes have reported that treatment with the glucose lowering drug metformin limits cardiovascular morbidity and mortality, independent from its glucose-lowering action.<sup>45</sup> This suggests that metformin might have direct cardioprotective properties. Many preclinical studies report beneficial effects of metformin on atherosclerosis development, myocardial IR-injury, and postinfarction remodeling.<sup>46</sup> In murine models of myocardial infarction, the administration of metformin, either before ischemia or at the onset of reperfusion, consistently limits infarct size. **Chapter 3** of this thesis gives a more detailed overview of the preclinical studies on the cardioprotective effects of metformin and the mechanisms underlying these effects. Briefly, the administration of metformin at reperfusion leads to activation of several kinases of the RISK pathway, including phosphatidylinositol-3-kinase (PI3K) and Akt, which in turn prevents opening of the mPTP. In addition to the RISK pathway, the cardioprotective effect of metformin is also critically dependent on activation of AMPK and eNOS and most recently, Paiva et al. showed that the cardioprotective effects of metformin are mediated through the increased intracellular formation of adenosine and subsequent adenosine receptor stimulation.<sup>47-49</sup> Although preclinical data convincingly show that metformin limits myocardial IR-injury, human data on a cardioprotective effect of metformin are scarce.<sup>46</sup> Therefore, in **chapter 4** of this thesis we investigated for the first time in humans whether short-term treatment with metformin reduces endothelial IR-injury *in-vivo* by measuring brachial artery flow mediated dilation (FMD) before and after prolonged ischemia and reperfusion of the forearm in healthy volunteers.

In addition, in **chapter 5** we investigate whether metformin can limit myocardial injury in the setting of CABG surgery.

#### **Dipyridamole**

The platelet aggregation inhibitor dipyridamole is registered for the secondary prevention of cerebrovascular events. Dipyridamole is a potent inhibitor of the hENT1.<sup>50,51</sup> Dipyridamole therefore leads to increased extracellular adenosine concentrations and subsequent adenosine receptor stimulation. In preclinical animals studies, and studies in healthy volunteers and patients undergoing coronary angioplasty (PCI), dipyridamole limits IR-injury.<sup>51-58</sup> In **chapter 6**, we tested whether administration of dipyridamole limits myocardial injury during CABG surgery.



## Ticagrelor

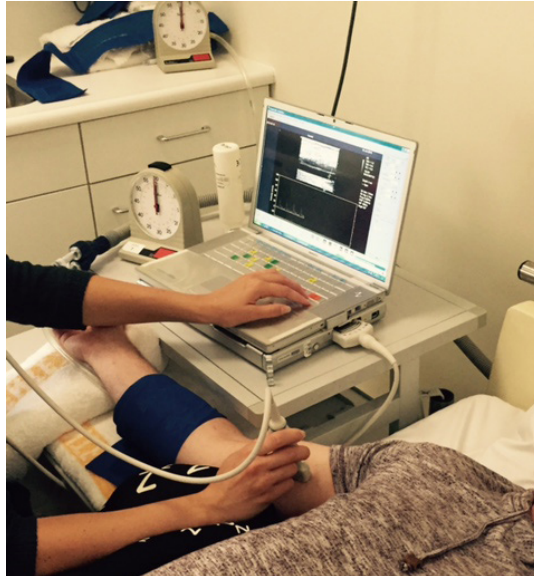
Ticagrelor is a novel direct-acting and reversibly binding P2Y<sub>12</sub> receptor antagonist. In the PLATO (PLATElet inhibition and patient Outcomes) trial, the administration of ticagrelor to patients with an acute coronary syndrome resulted in a striking reduction in the primary endpoint of death from vascular causes, myocardial infarction, or stroke compared to clopidogrel.<sup>59</sup> Moreover, all-cause mortality was reduced, and this was driven not only by vascular mortality, but also by fewer deaths attributed to sepsis.<sup>59</sup> In the past years, evidence has accumulated that ticagrelor inhibits ENT and therefore leads to a subsequent increase in extracellular adenosine concentration and adenosine receptor stimulation.<sup>60</sup> This mechanism has been proposed to mediate the effects of ticagrelor observed in the PLATO study. Most of the current evidence on the effect of ticagrelor on adenosine metabolism is derived from *in vitro* studies, or studies in patients in which the adenosine metabolism could be affected by other factors, such as co-medications. Therefore, in **chapter 7** we aimed to test the hypothesis that ticagrelor inhibits the ENT transporter in healthy humans *in vivo*. We used venous occlusion plethysmography of the forearm to measure the vasodilator response to local administered adenosine and to forearm ischemia, as validated surrogates for adenosine uptake inhibition.

## Experimental models used in this thesis

This paragraph summarizes the different models that we used in this thesis to study IR-injury and to provide mechanistic insights in humans.

### Flow mediated dilation following brachial artery occlusion

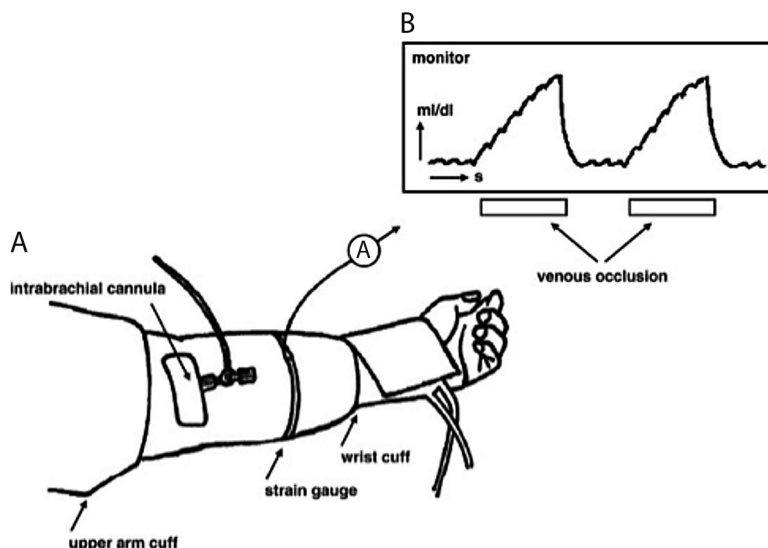
Flow mediated dilation (FMD) represents a non-invasive technique to measure endothelium-dependent vasodilation as a marker of endothelial function (figure 2).<sup>61</sup> The endothelial cells are highly susceptible to IR injury: during myocardial ischemia endothelial swelling and impaired endothelium-dependent relaxation can further impede proper tissue reperfusion.<sup>62</sup> FMD following forearm ischemia and reperfusion is a generally accepted model to study endothelial IR-injury in humans *in vivo*. Several previous studies have reported that twenty minutes of forearm ischemia followed by twenty minutes of reperfusion (induced by inflation/deflation of a pneumatic cuff around the proximal upper arm) impairs subsequent FMD of the brachial artery.<sup>63,64</sup> Furthermore, several studies demonstrated that this IR-injury-induced endothelial dysfunction could be prevented by (remote) ischemic preconditioning or postconditioning.<sup>63-66</sup> Also, pharmacological interventions (e.g. statins) significantly reduced endothelial IR-injury in this model.<sup>67</sup>



**Figure 2** Experimental set-up during brachial artery flow mediated dilation to measure endothelium-dependent vasodilation as a marker of endothelial function. A rapid inflation and deflation pneumatic cuff is used to induces forearm ischemia. In this thesis a 20 minutes ischemia-reperfusion protocol was used.

### Venous occlusion plethysmography

Another safe and well-validated technique to assess endothelial function is venous occlusion plethysmography (figure 3).<sup>68,69</sup> Administration of vaso-active drugs into the brachial artery results in a high local concentration in the forearm arterial bed. Inflation of an upper arm cuff to 40 mmHg allows for unhindered arterial inflow but hampers venous return. This results in swelling of the forearm. The rate at which this occurs can be measured by mercury-in-silastic strain gauges and correlates with forearm blood flow (FBF). To restrict blood flow measurement to skeletal muscle blood flow as much as possible, the circulation of the hand, which is mainly skin circulation, is excluded from the experimental preparation by inflation of a wrist cuff to 200 mmHg during each measurement. Intrabrachial administration of adenosine increases FBF due to a direct vasodilator effect. We and others have previously reported that concomitant administration of an ENT-inhibitor profoundly augments adenosine-induced vasodilation.<sup>70</sup> As such, we used adenosine-induced vasodilation as a surrogate marker for ENT inhibition to investigate the effects of ticagrelor.



**Figure 3** Illustration of the technique of venous occlusion plethysmography to measure forearm blood flow (A).

An intrabrachial cannula allows administration of drugs into the brachial artery. The rate of swelling of the forearm during venous occlusion is measured with a mercury-in-silastic strain gauge, which is connected to a computer (B), and is expressed in ml/min per dl of forearm volume. Reproduced with permission from Riksen et al., *Eur J Pharmacol.* 2008 May 13;585 (2-3):220-7.

### Coronary artery bypass grafting

The impact of ischemic conditioning has been widely studied in patients undergoing coronary artery bypass grafting (CABG).<sup>71-75</sup> During this intervention, to create a blood-less field, the aorta is cross-clamped to isolate the heart from the systemic circulation, which induces global myocardial ischemia. After surgical insertion of the grafts, the cross-clamp is removed from the aorta, a process which subjects the heart to acute global myocardial reperfusion. Therefore, CABG surgery results in myocardial IR-injury, which is reflected by a post-operative increase in plasma troponin, which is a biomarker for myocardial injury. The plasma troponin concentration in the first 24 hours after surgery is positively associated with a worse clinical outcome.<sup>76</sup>

Already in 1993, Yellon et al. were the first to apply ischemic preconditioning to patients scheduled for CABG surgery, by clamping the aorta for 2 minutes and unclamping the aorta for 2 minutes prior to the sustained global myocardial ischemia.<sup>77,78</sup> They found that those patients that received this form of preconditioning showed less peri-operative myocardial injury as evidenced by preserved ATP levels in ventricular biopsies<sup>54</sup> and lower serum troponin I concentrations.<sup>78</sup> In a study by Hausenloy et al., remote ischemic precondition consisting of three 5-minute cycles of right upper limb ischemia, induced by an automated cuff-inflator placed on the upper arm and inflated to 200 mmHg resulted in a significant 43% reduction in overall serum troponin-T release at 6, 12, 24 and 48 hours after surgery.<sup>72</sup> Since these pioneering clinical trials, several clinical studies have investigated ischemic preconditioning in the setting of CABG surgery.<sup>79</sup>

### **Post-ischemic recovery of contractile function in isolated human atrial trabeculae**

Coronary artery bypass grafting also offers the opportunity to harvest atrial tissue to measure in an ex-vivo model the effects of simulated ischemia and reperfusion on contractile function of isolated atrial trabeculae. This experimental set up was first described by the group of Yellon and has been used since then for investigating the effects of pre- and postconditioning of the human heart.<sup>80-82</sup> In summary, the right atrial appendage is harvested during cardiac surgery before the introduction of the extracorporeal circulation. After dissecting separate atrial trabeculae, these are suspended in an organ bath and linked to a force transducer. In this thesis a modified setup was used in which two trabeculae could be measured simultaneously.<sup>83</sup> After a recovery phase in which the trabeculae are superfused with a nutrient-supplemented and well-oxygenated solution, the trabeculae are exposed to 90 minutes of simulated ischemia, which is accomplished by removing oxygen and nutrients from the superfusate. Then reperfusion (during a period of 100 minutes) is simulated by restoring oxygen and nutrient supply to the trabeculae. Recovery of contractile function is used as surrogate marker for IR-injury.

## **Aim and outline of this thesis**

Most research on cardioprotection is performed in animal models. In this thesis, we aim to 1) develop novel models to study IR-injury in humans, 2) use existing human models of IR-injury to try to confirm beneficial effects of dipyridamole and metformin on IR-injury in humans in vivo, and 3) to provide more insight into the mechanism as to how metformin and ticagrelor could limit IR-injury.

In **chapter 2**, we aim to develop a novel human in vivo model of IR injury. We hypothesized that strenuous exercise increases plasma high-sensitive troponin levels in healthy volunteers, and that this can be prevented by Remote Ischemic Preconditioning (RIPC).

In **chapter 3** we give a more detailed introduction of pharmacological preconditioning with the glucose lowering drug metformin, mainly focusing on preclinical data and large observational clinical trials.

In **chapter 4** we test whether short-term treatment with metformin reduces endothelial IR-injury in healthy volunteers measured by flow mediated dilation following forearm ischemia.

In **chapter 5 and 6**, we move to the clinical setting and test the hypotheses that metformin and dipyridamole limit myocardial injury during CABG surgery.

Finally, in **chapter 7** we investigate whether ticagrelor blocks ENT transport at relevant plasma concentrations. This finding would provide an explanation for the beneficial effect of ticagrelor in patients with an acute myocardial infarction, over and above platelet aggregation inhibition.

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# 2

## The effect of remote ischemic preconditioning on exercise-induced plasma troponin I appearance in healthy volunteers

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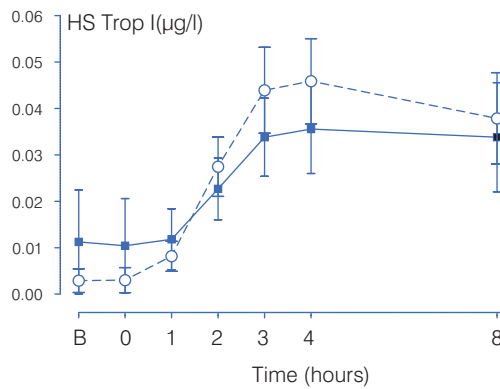
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Following prolonged endurance exercise (e.g. marathon running), circulating levels of cardiac troponin are increased in more than 50% of individuals.<sup>1</sup> Also after controlled laboratory-based exercise of shorter duration, plasma troponin concentration increases, but these data are sparse.<sup>2, 3</sup> Up to now, the underlying mechanism of troponin appearance after strenuous exercise in humans is unknown. Many theories have been posed, including exercise-induced increase in cardiomyocyte membrane permeability or cardiomyocyte injury due to ischemia reperfusion (IR).<sup>1</sup> In this study we aimed to investigate whether IR-injury contributes to the rise in circulating troponin after strenuous exercise in healthy volunteers. Remote ischemic preconditioning (RIPC) is a powerful strategy to limit IR-injury.<sup>4</sup> Therefore, in a randomized controlled cross-over study in healthy volunteers, we studied the effect of RIPC on exercise-induced troponin I release. If IR-injury contributes to the plasma appearance of troponin in this model, than exercise-induced troponin release can be used as a model of myocardial IR-injury in healthy volunteers.

The study was approved by the local ethics committee. After informed consent, twenty healthy volunteers (age  $22 \pm 4$  years, 10 male) participated. Before randomization, all volunteers underwent a maximal cycling test to determine maximum heart rate. Subsequently, in a crossover design, all volunteers performed a submaximal exercise test preceded by RIPC or a control intervention. The tests were separated by two weeks and the order was randomized. After 30 minutes of supine rest, subjects performed bicycle exercise at 80% of their maximum heart rate or heart rate reserve (whatever resulted in the highest target heart rate) for 70 minutes, immediately followed by cycling at 95% of maximal heart rate reserve until exhaustion or for a maximum of 15 minutes. The RIPC stimulus was applied during the 30 minutes immediately before the exercise test. RIPC consisted of three 5-min cycles of bilateral forearm ischemia, induced by inflation of an upper-arm cuff to 200 mm Hg, separated by 5 minutes of deflation. Five minutes after the last period of ischemia, the exercise test was performed. All experiments were performed in the morning, after a light breakfast and at least 24 hours of caffeine and alcohol abstinence. Forty-eight hours before each test, participants were instructed to abstain from strenuous exercise. On both study days, blood was sampled from an indwelling forearm catheter before, and at 0, 1, 2, 3, 4, and 8 hours after the exercise test to determine high sensitive troponin I (hs-troponin). N-terminal pro-B type natriuretic peptide (NT-Pro-BNP) was measured 1 and 3 hours post exercise. Creatine kinase (CK) was measured at  $t = 3$  hours post exercise. Results are described as mean  $\pm$  SE. The effect of RIPC on troponin release was tested with an ANOVA for repeated measures.

The workload and heart rate were not significantly different between exercise and RIPC+exercise (workload  $146 \pm 12$  vs.  $140 \pm 12$  Watt; heart rate  $168 \pm 1.5$  vs.  $169 \pm 1.4$  beats/min, respectively, all  $P > 0.05$ ). In both groups a consistent rise and fall in hs-troponin levels was found after exercise. There was no significant difference in hs-troponin levels between the groups with and without RIPC (figure 1). Also, post-exercise NT-Pro-BNP and CK levels were not different between both conditions (NT-pro-BNP  $t=1$  h exercise  $67.5 \pm 7.9$  vs. RIPC  $93.1 \pm 19.5$  ng/L; NT-pro-BNP  $t=3$  h exercise  $61.5 \pm 5.9$  vs. RIPC  $88.2 \pm 17.7$  ng/L; CK exercise  $323 \pm 85$  vs. RIPC  $431 \pm 118$  U/L;  $P > 0.1$  for all comparisons).



**Figure 1** Circulating hs-troponin I concentration (mean  $\pm$  SE) before (B) and after (0, 1, 2, 3, 4 and 8 h) cycling exercise with (open circles) or without (filled squares) preceding RIPC.

Our study is the first to measure hs-troponin levels on multiple time points after brief strenuous exercise in healthy volunteers. In accordance with previous studies of prolonged exercise, the plasma hs-troponin I concentration significantly increased in the majority of subjects with a peak concentration 4 hours post-exercise.<sup>3, 5</sup> RIPC did not alter exercise-induced hs-troponin release. This observation strongly suggests that myocardial IR-injury does not contribute to the exercise-induced increase in troponin. Therefore, this experiment cannot be used as a model to study IR-injury in humans in vivo.

A number of studies have demonstrated that strenuous exercise increases troponin levels in healthy individuals.<sup>1, 3</sup> The mechanism of this release, however, is still a matter of debate.<sup>1</sup> In cardiomyocytes, most troponin is bound to tropomyosin, and only a small amount resides in unbound form in the cytosol. Several mechanisms of

exercise-induced troponin release have been suggested, including an exercise-induced increase in myocardial sarcolemmal permeability, leading to leakage of unbound cytosolic troponin.<sup>1</sup> This increased membrane permeability can be due to mechanical stress, free oxygen radical formation or changes in pH levels.<sup>1, 2</sup> An alternative explanation might be stimulation of integrines due to myocardial stretch.<sup>6</sup> Stimulating integrines mediates the transport of intact troponin molecules out of the cardiomyocyte. Finally, troponin release can be due to sustained myocardial cell injury with cell death.<sup>5</sup> Troponins are also expressed in skeletal striated muscle, but since the amino-acid sequence of the skeletal and cardiac isoforms of troponin T and I are dissimilar, they are well-differentiable by monoclonal antibody-based assays.<sup>7</sup>

Should IR-injury contribute to the hs-troponin release after exercise, we would have expected RIPC to prevent this release, since RIPC consistently limits IR-injury in various situation of (myocardial) IR-injury.<sup>8, 9</sup> The observation that RIPC does not affect the appearance of troponin strongly argues against IR-injury to contribute significantly to the troponin release. In addition, we found no rise in NT-pro-BNP levels, suggesting no significant increase in intracardiac pressure. Hs-troponin release due to increased myocardial stretch is therefore unlikely. The mechanism responsible for the increased post-exercise hs-troponin levels still has to be elucidated. Taken together, the kinetics of the appearance of troponin, and the lack of protection by RIPC supports the concept that the troponin appearance is due to leakage of cytosolic troponin by mechanisms other than IR-injury.

In summary, we investigated whether myocardial IR-injury contributes to the exercise-induced increase in plasma hs-troponin I, using RIPC as a potent strategy to limit IR-injury. Based on our findings, IR-injury does not importantly contribute to the increase in hs-troponin levels after exercise as it cannot be prevented by RIPC. Strenuous exercise, therefore, is not a valid model to study myocardial IR-injury.



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# 3

## Metformin therapy in diabetes: the role of cardioprotection

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## Abstract

In patients with diabetes mellitus, the incidence of cardiovascular disease is increased, and the outcome following cardiovascular events is worse. The antihyperglycemic drug metformin appears to limit cardiovascular death in patients with type 2 diabetes. Indeed, preclinical studies have demonstrated that metformin limits (myocardial) ischemia and reperfusion injury, independent from its glucose-lowering effect. This cardioprotection is mediated by activation of the Reperfusion Injury Salvage Kinase (RISK) pathway, activation of AMPK and by an increased formation of adenosine. In addition, metformin can modulate several cardiovascular risk factors and reduces the development of heart failure in murine models. Consequently, treatment with metformin might potentially improve cardiovascular outcome in patients at risk for myocardial ischemia, even these patients do not have diabetes. In the current paper, we focus on the direct cardioprotective actions of metformin and the mechanisms that underlie these effects.

## Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide, accounting for nearly one third of all deaths.<sup>1</sup> In patients with diabetes mellitus, cardiovascular morbidity and mortality is even more pronounced. In part, this is explained by an accelerated development of atherosclerosis.<sup>2</sup> In addition, when suffering a myocardial infarction, the outcome is worse<sup>3-6</sup>, with also success rates of angioplasty and coronary artery bypass grafting being lower.<sup>7-9</sup> Patients with diabetes also have a high prevalence of congestive heart failure secondary to diabetic changes in the myocardium. This unique clinical entity, which is characterized by fibrotic changes in the myocardium and functional alterations in diastolic relaxation and ventricular compliance, is referred to as 'diabetic cardiomyopathy'.<sup>10</sup> The pathological changes occurring in diabetic cardiomyopathy include increased reactive oxygen species (ROS),<sup>11</sup> abnormal handling of calcium and increased release of inflammatory mediators.<sup>12</sup> Although in part related to myocardial infarction, this type of heart failure in diabetics can occur independent from cardiovascular risk factors and ischemia.<sup>12</sup> The prevalence of patients with diabetes and heart failure is rapidly increasing, and these patients have a worse outcome than heart failure patients without diabetes.<sup>13</sup> These observations, alongside the evidence that diabetic patients have a worse prognosis following myocardial infarction, suggest that the diabetic heart is more vulnerable to ischemic damage.

Therefore, novel strategies to prevent the development of atherosclerosis, and to increase the intrinsic tolerance of the myocardial cells to ischemia and reperfusion are urgently needed. In accordance with current guidelines, establishing early reperfusion is the primary therapy in patients with a myocardial infarction.<sup>14</sup> The beneficial effect of re-establishing coronary blood flow, however, is limited by a phenomenon called 'reperfusion injury'.<sup>14</sup> The process of reperfusion triggers various pathways, including oxidative stress, pH changes, calcium overload, an acute inflammatory response and metabolic changes, which cause tissue injury themselves, and contribute to the final myocardial infarct size. Many of these factors promote the opening of the mitochondrial permeability transition pore (mPTP), a critical determinant of cell death in the setting of acute ischemia reperfusion (IR)-injury.<sup>1, 15</sup> To further optimize the results of coronary angioplasty and coronary artery bypass grafting, strategies to limit IR-injury are needed. In the past two decades, both pharmacological and non-pharmacological interventions have been developed which can limit or prevent acute myocardial IR-injury.

As a nonpharmacological cardioprotective strategy, ischemic preconditioning (IPC) has received much attention for its potent infarct size-limiting effect since it was first reported by Murry et al. in 1986.<sup>16</sup> Murry demonstrated that short intermittent episodes of ischemia prior to a prolonged ischemic event potentially limit the final infarct size. Over the years, a tremendous amount of effort has been done to mimic the cardioprotective effect of IPC with pharmacological agents.<sup>17, 18</sup> Interestingly, several large studies have suggested that the glucose lowering drug metformin might have such a cardioprotective effect, independent of its glucose lowering effect.<sup>13</sup> In this article we aim to summarize these cardioprotective effects of metformin and the underlying mechanisms.

## **The effect of metformin on cardiovascular outcome in patients with type 2 diabetes**

Metformin, a biguanide glucose-lowering agent, is the first-line oral treatment option for patients with type 2 diabetes mellitus.<sup>19</sup> Metformin lowers plasma glucose levels by reducing insulin resistance, particularly in the liver and skeletal muscle cells. It suppresses hepatic gluconeogenesis, increases insulin sensitivity, enhances peripheral glucose utilization and has beneficial effects on lipid metabolism. The mechanisms underlying these effects have yet to be fully elucidated, although recent data have implicated AMP-activated protein kinase (AMPK) activation as an important mediator.<sup>20, 21</sup> One of the most significant breakthroughs in the understanding of the cellular mechanism of metformin was made in the early 2000s by two independent research groups reporting that metformin induces specific inhibition of complex 1 of the mitochondrial respiratory chain.<sup>22, 23</sup> It has been hypothesized that metformin activates AMPK by inhibiting complex 1 of the mitochondrial respiratory chain, resulting in a fall in the intracellular adenosine triphosphate (ATP) concentration and an increase in the adenosine monophosphate (AMP) to ATP ratio. In turn, AMPK activation leads to enhanced glucose uptake in skeletal muscle, stimulates oxidation of free fatty acids and inhibits glucose production by hepatocytes.<sup>24</sup> The AMPK hypothesis however, has recently been challenged by Foretz et al.<sup>25</sup> In their study they demonstrated that administration of metformin in mice suppressed hepatic gluconeogenesis directly by a decrease in hepatic energy state independent from AMPK activation.

Several large observational studies in patients with type 2 diabetes have reported that treatment with metformin limits cardiovascular morbidity and mortality independent from its glucose-lowering action.<sup>26</sup> In the United Kingdom Prospective Diabetes Study (UKPDS) treatment of diabetic patients with metformin was associated with lower mortality rates compared to treatment with sulphonylurea derivatives (SUD's).<sup>27</sup>

The study showed that patients with type 2 diabetes treated with metformin had a 36% lower all-cause mortality and a 39% lower risk of myocardial infarction compared with conventional treatment. This risk reduction was greater for metformin than for treatment with insulin or SUD's, despite similar glycaemic control. Also in patients with diabetes who have suffered from a myocardial infarction in the past, treatment with metformin is associated with a lower mortality rate than treatment with SUD's.<sup>28, 29</sup>

The reduction in cardiovascular mortality by metformin was confirmed by a systematic review and a recently published Cochrane analysis.<sup>30, 31</sup> Selvin et al concluded that treatment with metformin of overweight diabetic patients was associated with a decreased risk of cardiovascular mortality (pooled OR 0.74;95% CI, 0.62-0.89) compared with any other oral antidiabetic agent or placebo. The results for cardiovascular morbidity and all-cause mortality showed a similar trend, but this was not statistically significant. It needs to be mentioned however, that this analysis was restricted to the overweight patients.

A more recent meta-analysis was performed by Lamanna et al. in which they studied 35 clinical trials including 18,472 participants treated with metformin or comparator.<sup>32</sup> In this meta-analysis the overweight group and the non-overweight group were combined. In addition to patients with diabetes, they included studies on non-diabetic patients with HIV or polycystic ovary syndrome. In an overall analysis, metformin therapy when compared to an active comparator, did not produce any significant effect on the incidence of cardiovascular events. Monotherapy with metformin, however, induced a trend towards improved overall mortality, which was abolished when metformin was administered in addition to sulphonylurea derivatives.

The contradicting results of the recent meta-analysis and the striking beneficial effect of metformin in the UKPDS study, might be related to the fact that the UKPDS was performed in a much earlier time period where the treatment of other cardiovascular risk factors and the treatment of acute coronary events was inferior to the treatment in the present era. Therefore, in the current era establishing a beneficial cardioprotective effect of metformin on top of its glucose-lowering effect is a greater challenge.

Laboratory studies, animal studies, and studies in healthy volunteers and patients with diabetes have provided evidence that metformin modulates several risk factors for atherosclerosis, increases the intrinsic tolerance of the myocardium to ischemia and reperfusion, and attenuates the subsequent development of heart failure. We will consecutively discuss these studies and focus on the direct cardioprotective effects of metformin.



## Effect of metformin on other cardiovascular risk factors

In addition to its glucose lowering effect, metformin therapy also has beneficial effects on other cardiovascular risk factors. Patients with diabetes exhibit an atherogenic lipid profile characterized by hypertriglyceridemia, decreased levels of high-density lipoprotein (HDL) cholesterol and elevated levels of small, dense atherogenic low density lipoprotein (LDL) cholesterol particles.<sup>33, 34</sup> Free fatty acid levels are increased, and consequently, hepatic production of very low density lipoprotein (VLDL) is increased and clearance of VLDL particles is reduced.<sup>35, 36</sup> Metformin seems to improve the lipid profile by lowering triglyceride levels, total cholesterol and LDL cholesterol levels while maintaining or increasing HDL levels.<sup>37-42</sup> By lowering glucose levels metformin also seems to reduce oxidative stress and lipid oxidation.<sup>43, 44</sup>

An increase in bodyweight is also associated with an increased risk for cardiovascular disease and is deemed an independent risk factor for CVD by the Framingham Heart Study.<sup>45</sup> Metformin reduces bodyweight by enhancing carbohydrate utilization in the gastrointestinal tract, adverse gastrointestinal side effect, carbohydrate malabsorption, and/or through associated anorexia.<sup>37</sup>

Another suggested beneficial effect of metformin is its ability to lower blood pressure. Even a small elevation in blood pressure significantly increases death from cardiovascular disease and risk for myocardial infarction, stroke and congestive heart failure in patients with diabetes.<sup>46</sup> Therefore even a minimal reduction in blood pressure during treatment with metformin may contribute to a significant decrease in diabetes related morbidity and mortality. Several studies indicate an antihypertensive effect of metformin in animals and humans.<sup>38, 47-52</sup> Data regarding the effect of metformin on blood pressure however, are not entirely consistent. In a study by Nagi et al metformin had no effect on blood pressure in patients with diabetes and a recent study by He et al showed the same results for non-diabetics.<sup>53, 54</sup>

Platelet aggregation plays an important role in the pathophysiology of myocardial infarction. Metformin has been shown to reverse platelet hyperaggregation, but this effect is more pronounced in animal experimental models than in humans.<sup>55</sup> Gin et al was able to demonstrate that in patients with diabetes mellitus treated with insulin, administration of metformin decreased the maximum platelet aggregation induced by adenosine diphosphate (ADP) in vitro.<sup>56</sup> Another study however failed to demonstrate any effect on platelet aggregation or platelet aggregation induced by ADP.<sup>53</sup>

Another risk factor for cardiovascular arterial thrombotic events is an elevated fibrinogen level.<sup>57</sup> In patients with insulin resistance, fibrinolysis is reduced, which seems correlated to increased levels of plasminogen activator inhibitor (PAI)-1.<sup>58</sup> Metformin appears to reduce PAI-1 levels in patients with and without diabetes, hereby reducing the risk of developing cloth formation.<sup>59-61</sup>

Endothelial dysfunction, as characterized by an impairment in endothelium dependent relaxation and reduced nitric oxide (NO) bioavailability, is an early characteristic of atherogenesis. Studies have shown that treatment with metformin improves endothelium-dependent vasodilation by increased availability of NO.<sup>62</sup>

All of the abovementioned beneficial effects of metformin on cardiovascular risk factors could potentially lead to slowing down the process of atherosclerosis and therefore decrease the incidence of cardiovascular events.

## Direct cardiovascular effects of metformin

### Effect of metformin on ischemia reperfusion injury

The administration of metformin, either before the ischemic stimulus or at the moment of coronary reperfusion, profoundly reduces infarct size in murine models of myocardial infarction. Already in 1988, Charlon et al showed that oral administration of metformin could reduce infarct size in rats.<sup>63</sup> Solso et al also showed in a rat isolated perfused heart model that the administration of a single dose of metformin 24 hours before transient coronary artery occlusion reduced infarct size.<sup>64</sup> In subsequent studies in isolated rat hearts, coronary perfusion with metformin during the first 15 minutes of reperfusion reduced infarct size with approximately 40-50%.<sup>65-67</sup> This was also confirmed in in-vivo studies in rats and mice.<sup>66, 68</sup> Recently, several intracellular mechanisms that mediate this cardioprotective effect have been discovered.

### Reperfusion Injury Salvage Kinase (RISK) pathway

Opening of the mitochondrial permeability transition pore (mPTP) during (early) reperfusion is a pivotal mechanism of ischemia-reperfusion (IR)-injury. This non-selective pore in the mitochondrial inner membrane is closed during ischemia. Immediately after reperfusion, the mPTP opens, which will induce ATP depletion and release of pro-apoptotic factors, including cytochrome C, ultimately leading to necrosis and apoptosis.<sup>69, 70</sup> It has been demonstrated that the administration of metformin at reperfusion leads to activation of several kinases of the Reperfusion Injury Salvage Kinase (RISK) pathway, including phosphatidylinositol-3-kinase (PI3K) and Akt, which in turn can prevent opening of the mPTP.

In an isolated rat heart model, Bhamra et al. found that the administration of metformin during the first 15 minutes of reperfusion reduced infarct size in nondiabetic Wistar rats and type 2 diabetic Goto-Kakizaki rats.<sup>65</sup> Metformin also induced a significant increase in Akt phosphorylation after reperfusion. Concomitant administration of LY294002, a PI3K inhibitor, prevented Akt phosphorylation and abolished the protective effect on infarct size. In isolated rat cardiomyocytes, incubation with metformin prevented mPTP opening by activation of the PI3K/Akt pathway. The authors concluded that the protective effect of metformin against myocardial IR-injury is PI3K dependent and that the effect of metformin on Akt phosphorylation is similar in both nondiabetic and diabetic rats.

### AMPK

In addition to the RISK pathway, the cardioprotective effect of metformin is also critically dependent on activation of AMPK. AMPK is a major regulator of energy balance in the cell. Environmental stress including exercise, starvation, inflammation and hypoxia increases AMPK activity. AMPK also fulfils a crucial role in preserving myocardial viability during myocardial infarction. By increasing ATP synthesis and lowering ATP utilization, AMPK functions to maintain normal cellular energy stores during ischemia.<sup>24</sup> Calvert et al. showed that the protective effect of metformin is AMPK dependent.<sup>68</sup> Immediately after the onset of myocardial ischemia, phosphorylation of AMPK occurs, and remains active for more than 24 h following reperfusion in a murine model of coronary artery occlusion. Pretreatment with a very low dose of metformin increased the phosphorylation of AMPK in hearts not exposed to ischemia as well as in hearts exposed to ischemia reperfusion. Metformin failed to provide protection in cardiac-specific AMPK $\alpha$ 2 dominant-negative transgenic mice, demonstrating that AMPK activation is essential for metformin-induced protection. In an additional experiment, it was shown that metformin increased eNOS phosphorylation via activation of AMPK and that eNOS activation was also indispensable for its cardioprotective action.<sup>68</sup> Yellon's group also studied the involvement of AMPK in metformin-induced cardioprotection.<sup>67</sup> In a rat isolated heart model, metformin was added for 15 min at the onset of reperfusion alone or with the AMPK inhibitor Compound C. Metformin significantly reduced infarct-size, which was completely abolished by adding Compound C simultaneously. Interestingly, delayed administration of Compound C after 5 minutes of reperfusion did no longer block the protective effect of metformin. The authors concluded that the cardioprotective effect of metformin was mediated by AMPK activation very early during reperfusion

## Adenosine

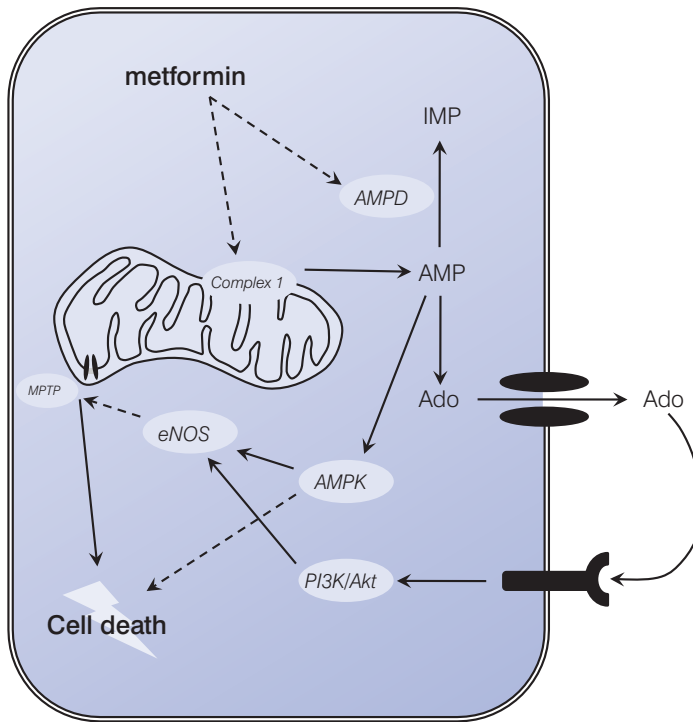
Most recently, the endogenous nucleoside adenosine has been shown to be involved in metformin-induced cardioprotection. Metformin induces phosphorylation of AMPK, which is mediated, at least in part, by an increase in the cytosolic AMP concentration.<sup>71</sup> The concentration of free AMP in the cytosol is also a major determinant of the intracellular formation of adenosine. It is well known that adenosine receptor stimulation limits infarct size by activation of the RISK pathway.<sup>17, 72</sup> Therefore, Paiva et al. hypothesized that adenosine receptor stimulation contributes to the infarct size limiting effect of metformin.<sup>66</sup> In isolated perfused nondiabetic rat hearts, perfusion with metformin during the first 15 minutes of reperfusion limited infarct size. Both 8-p-sulfophenyltheophylline (a nonspecific adenosine receptor blocker) and nitrobenzylthioinosine (transmembranous adenosine transport inhibitor) completely prevented the protective effect, suggesting that intracellular formation of adenosine, and subsequent adenosine receptor stimulation mediates the protective effect of metformin.

In conclusion, metformin potently reduces myocardial infarct size by activation of multiple, and probably parallel, intracellular pathways. These pathways include activation of the RISK pathway, activation of AMPK and eNOS and increased adenosine receptor stimulation, resulting in the prevention of MmTP opening at reperfusion. These signaling pathways are illustrated in Fig. 1.

## Effect of metformin on cardiac remodelling and heart failure

In patients suffering a myocardial infarction, the prognosis is determined not only by the final infarct size, but also by long-term changes that occur in the myocardium following an infarction. Following IR-injury, but also in patients suffering from volume overload (e.g. due to valvular disease), pressure overload (e.g. due to hypertension) and diabetic cardiomyopathy, a complex cascade of events occurs in the myocardial cells, including inflammation and fibrosis, ultimately leading to changes in size, shape and function of the heart, a process which has collectively been termed 'remodeling'.<sup>73, 74</sup> In time this cardiac remodelling will lead in a significant part of the patients to clinically overt heart failure. Several experimental animal studies showed that metformin, in addition to limiting IR-injury, can also limit the process of cardiac remodelling and the development of heart failure.<sup>75</sup>

Gundewar et al have shown in a series of experiments that metformin significantly improves left ventricular function and survival in a murine model of heart failure.<sup>76</sup> The administration of a single dose of metformin at reperfusion following 60 minutes of coronary artery occlusion reduced infarct size, but did not prevent the development of severe cardiomyopathy. In contrast, when metformin was administered daily for a



**Figure 1** Inhibition of complex 1 increases the AMP concentration.

This activates AMPK and increases the formation of adenosine. AMPK activation increases tolerance against ischaemia and reperfusion and, by phosphorylation of eNOS, prevents MPTP opening at reperfusion. In addition, increased adenosine receptor stimulation activates the PI3K/Akt pathway, which contributes to eNOS activation (Reproduced with permission from El Messaoudi et al, *Curr Opin Lipidol.* 2011;22:445-453).

period of 4 weeks, the development of cardiac hypertrophy was limited and heart function was preserved. In a model of permanent coronary artery ligation, treatment with metformin did not reduce infarct size and did not affect ejection fraction, although this treatment increased overall survival.

In their rat model of permanent coronary artery ligation, Yin et al have recently shown that the long term administration of metformin preserves left ventricular function.<sup>77</sup> In this study, animals were randomly allocated to the treatment with normal water or metformin-containing water (250 mg/kg/day). In the rats that received permanent

coronary artery ligation, infarct size was significantly smaller after 12 weeks of metformin treatment compared to the control group. Moreover, metformin treatment resulted in less left ventricular dilatation and preservation of left ventricular ejection fraction compared with the control group. In addition, in a dog model of cardiac pacing-induced heart failure and in a mouse model of heart failure due to thoracic aortic constriction, the chronic administration of metformin attenuated the hemodynamic and structural changes that were characteristic of the development of heart failure.<sup>78, 79</sup> In contrast, metformin did not prevent heart failure development in a rat model of volume overload-induced heart failure.<sup>80</sup>

Probably, several mechanisms contribute to this protective effect. Metformin is thought to improve myocardial mitochondrial respiration and ATP synthesis by an underlying mechanism requiring the activation of AMPK and its downstream mediators eNOS and peroxisome proliferator-activated receptor- $\gamma$  coactivator- (PGC) 1 $\alpha$ .<sup>76</sup> Also, metformin seems to reduce collagen expression that occurs after coronary artery ligation.<sup>77</sup> Yin et al also suggested that the beneficial effect of metformin on post-myocardial infarction remodelling could be attributed to a decrease in plasma insulin concentration, given the observation that hyperinsulinemia is associated with exacerbation of cardiac remodelling.<sup>77, 81</sup>

## Alternative glucose lowering drugs and cardioprotection

Interestingly, metformin is not the only glucose-lowering agent that can modulate (myocardial) IR-injury. Sulphonylurea derivatives (SUDs) do not induce cardioprotection, but have been shown to prevent the cardioprotective effect of ischemic preconditioning and pharmacological preconditioning, most likely due to prevention of opening of the mitochondrial ATP-sensitive K<sup>+</sup> channel.<sup>28, 29, 82-84</sup> This effect of SUD's might contribute to the findings that patients who are treated with a combination of SUD and metformin appeared to have a worse outcome on mortality as compared with those who are treated with metformin alone.<sup>32</sup> A recent national cohort study of veterans initiating oral treatment for diabetes mellitus found that sulfonylurea use was associated with an increased hazard of acute myocardial infarction, stroke, or death compared with metformin use. The findings do not clarify whether the difference in cardiovascular disease risk is due to harm from sulfonylureas, benefit from metformin, or both.<sup>27</sup> Recent comparative effectiveness reviews and meta-analyses concluded that metformin was associated with a slightly lower risk for all-cause mortality compared with sulfonylureas, but results were inconsistent and imprecise.<sup>30, 85, 86</sup> This study provides further evidence of a risk difference in cardiovascular outcomes for sulfonylurea and metformin users. Insulin, on the other hand, profoundly protects the

myocardium from IR-injury via activation of the RISK pathway.<sup>87</sup> Similarly, the novel antihyperglycemic glucagon like-peptide-1 ( GLP-1) analogues and the dipeptidyl-peptidase-4 (DPP-4) inhibitors have also been shown to exert direct cardioprotective effects in murine models of myocardial infarction. Very recently, the GLP-1 analogue exenatide was tested in patients suffering an acute ST-segment elevation myocardial infarction.<sup>88</sup> It appeared that a six-hours intravenous administration of exenatide, commencing immediately before reperfusion, significantly reduced infarct size, measured with cardiac magnetic resonance. Since this is outside the scope of our review, we would like to refer the reader to contemporary reviews on these alternative glucose-lowering drugs.<sup>89-91</sup>

## Conclusion

Animal studies have provided consistent evidence that metformin can limit myocardial ischemia reperfusion injury and infarct size. Metformin therapy also seems to attenuate postinfarction cardiac remodelling. In addition, metformin can modulate several risk factors for atherosclerosis, independent of glucose control, although these findings are less consistent. The most likely underlying mechanisms of cardio-protection are activation of the RISK-pathway, activation of AMPK, and increased adenosine receptor stimulation. Currently, several randomized clinical trials are being performed to explore the cardioprotective effect of metformin in nondiabetic patients suffering a myocardial infarction<sup>92</sup> and in nondiabetic patients undergoing cardiac surgery (ClinicalTrials.gov registration number NCT01439723). Metformin is cheap, and its longterm safety has been well established. Therefore, a positive result from these clinical studies could result in a quick implementation of metformin in clinical management to prevent and treat cardiovascular events, also in patients without diabetes mellitus.

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# 4

## Impact of metformin on endothelial ischemia-reperfusion injury in humans in vivo: a prospective randomized open, blinded-endpoint study

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## Abstract

**Introduction:** Large prospective studies in patients with type 2 diabetes mellitus have demonstrated that metformin treatment improves cardiovascular prognosis, independent of glycemic control. Administration of metformin potentially limits infarct size in murine models of myocardial infarction. This study examined, for the first time in humans, whether metformin limits ischemia-reperfusion (IR) injury *in vivo* using a well-validated forearm model of endothelial IR-injury.

**Methods:** Twenty-six healthy volunteers (age  $41\pm 6$  years, 10 male/16 female) were randomized between pretreatment with metformin (500 mg three times a day for 3 days) or no treatment in a Prospective Randomized Open Blinded Endpoint study. Brachial artery flow mediated dilation (FMD) was measured before and after 20 minutes of forearm ischemia and 20 minutes of reperfusion. FMD analysis was performed offline by investigators blinded for the treatment arm.

**Results:** Baseline FMD did not differ between metformin pretreatment and no pretreatment ( $6.9\pm 3.6\%$  and  $6.1\pm 3.5\%$ , respectively,  $p=0.27$ ,  $n=26$ ). FMD was significantly lower after forearm IR in both treatment arms ( $4.4\pm 3.3\%$  and  $4.3\pm 2.8\%$ , respectively,  $P<0.001$  in both conditions). A linear mixed model analysis revealed that metformin treatment did not prevent the decrease in FMD by IR.

**Conclusion:** A 3 day treatment with metformin in healthy, middle-aged subjects does not protect against endothelial ischemia reperfusion injury, measured with brachial artery FMD after forearm ischemia. Further studies are needed to clarify what mechanism underlies the cardiovascular benefit of metformin treatment.



## Introduction

Despite optimal reperfusion strategies, morbidity and mortality remain significant in patients suffering an acute myocardial infarction. Therefore, much effort is put into developing novel strategies to limit ischemia-reperfusion (IR) injury.<sup>1</sup> Interestingly, large observational and intervention studies have shown that overall cardiovascular mortality is lower in patients with type 2 diabetes mellitus who are treated with metformin, than in patients treated with alternative glucose-lowering drugs, despite similar glycemic control.<sup>2-6</sup> This observation suggests that metformin has direct cardioprotective effects.<sup>7</sup> Indeed, in murine models of myocardial infarction, performed in diabetic as well as in non-diabetic animals, administration of metformin limits myocardial infarct size.<sup>8-11</sup> This cardioprotective effect is mediated by activation of adenosine monophosphate activated protein kinase (AMPK) and adenosine receptor stimulation.<sup>9, 10</sup> Whether metformin treatment also directly protects against IR injury in humans is currently unknown.

Myocardial IR also induces endothelial dysfunction, causing endothelial swelling and impaired endothelium-dependent relaxation, which can further impede proper tissue reperfusion.<sup>12</sup> Several strategies, including ischemic preconditioning and postconditioning have been reported to limit IR-induced endothelial dysfunction in healthy humans.<sup>13, 14</sup>

In this study, we investigate for the first time in humans whether metformin limits endothelial IR injury *in vivo* by measuring flow mediated dilation (FMD) of the brachial artery before and after prolonged ischemia and reperfusion of the forearm. We test the hypothesis that short-term pretreatment with metformin limits endothelial IR injury in humans *in vivo*.

## Methods

### Ethics Statement

The protocol is approved by the Institutional Review Board of the Radboud University Medical Centre, and was performed in the Radboud University Medical Centre in compliance with the recommendations of the Declaration of Helsinki. All patients signed for informed consent before participation. The study is registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01610401). The authors confirm that all ongoing and related trials for this drug/intervention are registered.



## Participants

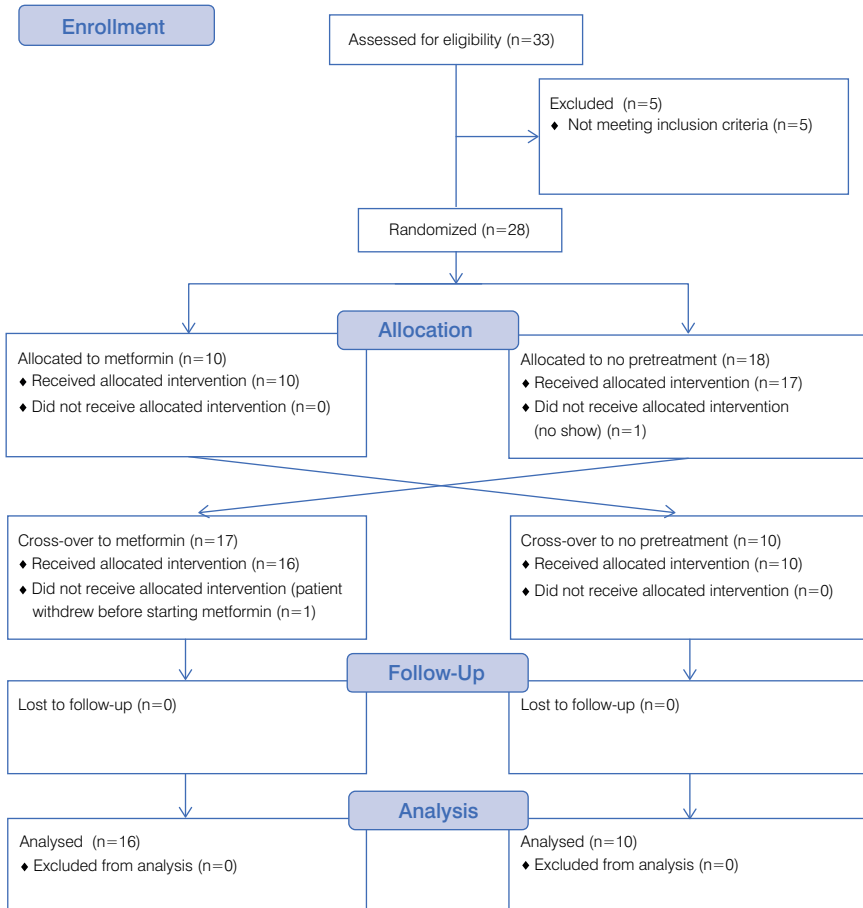
We included 28 healthy, non-smoking adult volunteers in this study. All subjects were free of cardiovascular disease, diabetes mellitus, hypertension (systolic blood pressure  $\geq 140$  and/or diastolic  $\geq 90$  mmHg) and hypercholesterolemia (random total cholesterol  $> 6.5$  mmol/L). We also excluded professional athletes and those who were taking concomitant medication. Oral contraceptive use by female participants was permitted and these females were asked to continue their contraceptive throughout the study to maintain stable hormone levels. Females not on oral contraceptives were measured at identical times in their menstrual cycle, to exclude any confounding effects of circulating hormones on endothelial function.<sup>15, 16</sup> Two participants withdrew during the study. Therefore, 26 subjects finished the trial protocol (Figure 1).

## Experimental Design

In a prospective randomized open blinded end-point (PROBE) study, subjects were allocated to treatment with either metformin 500 mg (Mylan, Bunschoten, The Netherlands) three times a day for 3 days, to ensure a steady state plasma concentration, or no pretreatment (figure 1). Simple random allocation was performed by an independent researcher by dice-throwing for each individual patient (even number: starting with metformin; uneven number: starting with no treatment). The last dose of metformin was given approximately 3 hours before the experiments. Subjects attended our laboratory twice, separated by at least 14 days to prevent any cross-over effect of metformin. Brachial artery endothelial function was measured with flow-mediated dilation (FMD) in the right arm, before and after 20 minutes of forearm ischemia. Forearm ischemia was induced by inflating a pneumatic cuff around the upper arm for 20 minutes and this was followed by 20 minutes of reperfusion. Patient recruitment was performed between May 15<sup>th</sup> 2012 and October 4<sup>th</sup> 2012. The first patient was included on May 15<sup>th</sup> 2012 and the last visit of the last patient was November 20<sup>th</sup> 2013.

## Measurements

All subjects had to abstain from caffeine consumption and strenuous exercise for at least 24 hours before the measurement. Measurements were performed after an overnight fast of at least 6 hours. Before the test, venous blood was taken to assess metformin and caffeine levels. After removing phospholipids and proteins with HybridSPE-phospholipids columns (Supelco), the plasma metformin concentration was determined with LC-MS/MS, using an Accela U-HPLC (Thermo Fischer Scientific) coupled to a TSQ Vantage (Thermo Fisher Scientific) triple quadrupole mass spectrometer. The compounds were separated on a Zorbax HILIC Plus (100x2.1 mm, 3.5  $\mu$ m particle size; Agilent Technologies). As internal standard we used metformin-d6 (Toronto Research Chemicals Inc.) The elution gradient was as follows: 0 min, 100%



**Figure 1** Consort 2012 flow diagram of the study.

B; 5 min, 50% B; and 6 min, 100% B. Solvent A consisted of 2 mM  $\text{NH}_4\text{format}+0.1\%$  formic acid in  $\text{H}_2\text{O}$  and Solvent B consisted of 2mM  $\text{NH}_4\text{format}+0.1\%$  formic acid in 90% Acetonitril. The column temperature was set at  $40^\circ\text{C}$ , and the flow rate was  $200\mu\text{l}/\text{min}$ . The effluent from the U-HPLC was passed directly into the electrospray ion source. Positive electrospray ionization was achieved using a nitrogen sheath gas with ionization voltage at 3500 Volt. The capillary temperature was set at  $290^\circ\text{C}$ . Detection of metformin and the internal standard was based on isolation of the protonated molecular ion,  $[\text{M} + \text{H}]^+$  and subsequent MS/MS fragmentations and a

selected reaction monitoring (SRM) were carried out. The following SRM transitions were used: for metformin  $m/z$  130,1 (parent ion) to  $m/z$  60,1 and 71,1 (both product ions) and for d6-metformin  $m/z$  136,1 (parent ion) to  $m/z$  77,1 (product ion).

Plasma caffeine concentrations were determined by use of reversed phase HPLC with UV detection set at 273 nm, as previously described.<sup>17</sup>

*Flow mediated dilation (FMD).* Endothelium-dependent vasodilation was assessed using FMD according to recent guidelines.<sup>18</sup> Participants rested in the supine position in a temperature-controlled room (22°C) for at least 15 minutes to allow baseline assessment of heart rate and blood flow. The subjects were tested at the same time of day to prevent diurnal variation in FMD responses. Mean arterial pressure was determined using a manual sphygmomanometer placed around the left arm.

To examine brachial artery FMD, the right arm was extended and positioned at an angle of ~80° abduction from the torso. A rapid inflation and deflation pneumatic cuff (D.E. Hokanson, Bellevue, WA) was placed distal to the olecranon process to provide an ischemic stimulus distal from the brachial artery. A 10-MHz (T3000, Terason, Aloka, UK) multi-frequency linear array probe attached to a high-resolution ultrasound machine was used to perform imaging. The brachial artery was imaged in the distal third of the upper arm. Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. A continuous Doppler velocity assessment was obtained simultaneously, and data were collected using the lowest possible insonation angle (always <60°), which did not vary during each study.<sup>18</sup> After a resting period of at least 15 minutes, 1 minute of baseline recording of the arterial diameter and velocity was performed. Subsequently, the occlusion cuff was inflated to 220 mmHg for 5 minutes. The arterial diameter and velocity recordings were restarted at least 30 seconds before cuff deflation and continued for at least 3 minutes after deflation. Peak arterial diameter and flow, and the time to reach this peak after cuff deflation, were recorded. Subsequently, the rapid inflation/deflation pneumatic cuff was positioned proximally around the upper arm to provide an occlusion for 20 minutes, so that the brachial artery was within the ischemic zone and was exposed to IR. The cuff was inflated for 20 minutes to 220 mmHg, which was followed by 20 minutes of reperfusion. Finally, the FMD measurement was repeated 20 minutes after reperfusion. All measurements were performed by the same, well-experienced sonographer who was blinded for the treatment allocation.

### Brachial artery diameter and blood flow analysis

Analysis of the brachial artery diameter was performed by an investigator who was blinded for the experimental treatment, using custom-designed edge-detection and wall-tracking software, which is independent of investigator bias.<sup>19</sup> Baseline data were calculated across the 1 minute preceding cuff inflation. Following cuff deflation, peak diameter was automatically detected according to an algorithm as described in detail elsewhere.<sup>20</sup> Within-subject reproducibility of the FMD using this semi-automated software is 6.7-10.5% (coefficient of variation).<sup>21</sup> Post-deflation shear rate data, derived from velocity and diameter measures, was used to calculate the area under the shear rate curve (SR<sub>AUC</sub>).

### Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 16). Data are presented as mean±SD unless stated otherwise. Baseline parameters between testing days were compared by paired *t*-tests.

In a previous study from our laboratory, IR injury reduced FMD with 2.6% with a SD of 3.7%. Assuming a correlation coefficient of 0.7 in our study, given the cross-over design, the expected SD of the effect of IR on FMD in our study therefore equals 2.85. With *n* = 26 subjects, we will be able to detect a difference of 1.65% with a power of 80% and a type I error probability of 5%, which is a relevant difference.

In order to evaluate the impact of IR on endothelial function (measured as FMD), and whether metformin can (partially) prevent endothelial IR, we employed a linear mixed model analysis and a two-way repeated measures ANOVA. Furthermore, according to a recent study by Atkinson *et al.*, inadequate scaling for FMD would be present if the upper confidence limit of the regression slope of the relationship between logarithmically transformed base diameter and peak diameter is less than one.<sup>22</sup> In such an event, FMD% is not an appropriate measure to estimate endothelial function. We checked our data for this phenomenon, and subsequently performed the allometric modelling solution proposed by Atkinson *et al.*<sup>22</sup> Subsequently, the FMD-values were re-analysed with a linear mixed model analysis with random factor subject and fixed factors IR (pre *versus* post), intervention (metformin *versus* no treatment), but also whether the type of intervention was associated with the different impact of IR on the change in FMD (i.e. interaction IR\*intervention). We used a Kolmogorov-Smirnov test to demonstrate a normal distribution of our outcome measures (FMD% and allometrically scaled FMD; *P*>0.1). The level of statistical significance was set at 0.05.

## Results

Baseline characteristics are presented in Table 1. All values were within the normal range.

**Table 1** Baseline characteristics.

Variable	Value
Age (yrs)	41.3±6.4
Body weight (kg)	74±13
Height (cm)	174±8
BMI (kg/m <sup>2</sup> )	24.2±2.9
Systolic blood pressure (mmHg)	120±9
Diastolic blood pressure (mmHg)	75±6
Creatinine (μmol/L)	73±13
MDRD-GFR (mL/min/1.73m <sup>2</sup> )	85±7
Glucose (mmol/L)	4.5±0.3
Cholesterol (mmol/L)	4.7±0.8

BMI; body mass index, MDRD; modification of diet in renal disease, GFR; glomerular filtration rate

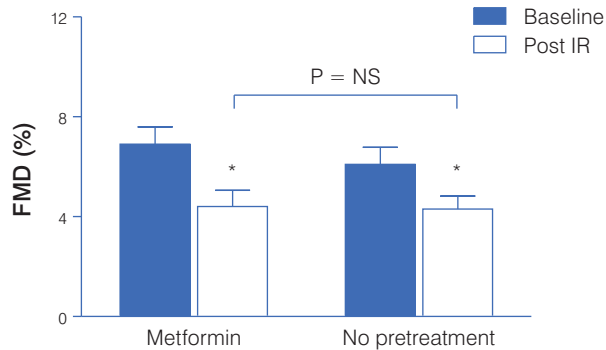
The metformin plasma concentration immediately before the experiment averaged 1357±588 ng/ml and were all within the therapeutic range (494 – 3237 ng/ml). Median plasma caffeine concentration was 0.12 (range 0-2.11 ) mg/dl, with 7 subjects >1 mg/dl. An overview of the FMD measurements is presented in table 2. There were no serious adverse events during the trial. At baseline, we found no differences in brachial artery characteristics (i.e. baseline diameter, time-to-peak diameter, and shear rate area-under-the-curve) between both testing days (Table 2). Baseline FMD% did not differ between metformin pretreatment and no pretreatment (6.9±3.6% and 6.1±3.5%, respectively,  $p=0.27$ ).

The IR protocol induced a significant increase in baseline brachial artery diameter and a decrease in shear rate stimulus that was not affected by metformin treatment (Table 2). Both in absence as well as in presence of metformin, brachial artery FMD% was significantly lower after forearm IR (4.4±3.3% and 4.3±2.7% respectively,  $p<0.01$  in both conditions). A two-way repeated measures ANOVA revealed that metformin treatment did not affect the decrease in FMD by IR (Figure 2;  $p=0.52$ ). Subsequent linear mixed model analysis, in which we included baseline diameter and  $SR_{AUC}$  as covariates to correct for the changes in these parameters after IR, confirmed our

**Table 2** Brachial artery characteristics before and after ischemia-reperfusion (IR) when preceded by metformin pretreatment or no pretreatment (n=26). Data is presented as mean±SD.

	No pretreatment		Metformin		P-values		
	Baseline	IR	Baseline	IR	IR	Metformin	IR*Metformin
FMD (%)	6.1±3.5	4.3±2.7	6.9±3.6	4.4±3.3	<0.001	0.52	0.23
FMD (mm)	0.19±0.09	0.15±0.09	0.22±0.08	0.15±0.11	<0.001	0.60	0.09
Diameter (mm)	3.5±0.9	3.8±0.9	3.5±0.8	3.7±0.9	<0.001	0.52	0.15
Time-to-peak diameter (s)	49±16	54±25	54±17	48±23	0.94	0.92	0.17
SR <sub>AUC</sub> (s, 10 <sup>3</sup> )	30.2±11.3	21.8±11.7	28.4±11.2	21.1±9.5	<0.001	0.51	0.86

FMD: flow mediated dilation; FMD (%) percent change to baseline; FMD (mm) absolute change to baseline; IR: ischemia-reperfusion; SR<sub>AUC</sub>: shear rate area under the curve.



**Figure 2** Brachial artery flow mediated dilation (presented as percentage change from baseline ±SE) before (black) and after (white) 20-minutes for forearm ischemia and 20-minutes reperfusion preceded by pretreatment with metformin or no pretreatment.

primary finding, in that the decrease in FMD% after IR was not altered by 3-days of metformin intake. A limited number of subjects (n=7) had a caffeine concentration >1 µg/ml. Exclusion of these subjects did not change our conclusion (data not shown).

## Discussion

Our current study is the first to investigate whether metformin limits IR-injury in humans *in vivo*. Using a well-validated model of forearm endothelial IR-injury in healthy middle-aged subjects, we demonstrated that a three day treatment with metformin does not protect against IR-induced endothelial dysfunction.

Our hypothesis that metformin ameliorates IR-injury is based on previous epidemiological studies and clinical trials in patient with diabetes and on preclinical animal studies. First, the United Kingdom Prospective Diabetes Study (UKPDS) reported that patients with type 2 diabetes mellitus who were treated with metformin had a significant lower cardiovascular mortality than patients treated with alternative glucose-lowering drugs, despite similar glycemic control.<sup>2</sup> Secondly, recent experimental studies in mice and rats showed that acute administration of metformin potentially reduced myocardial infarct size. In diabetic and nondiabetic mice, administration of a single dose of metformin either before ischemia or at the moment of coronary reperfusion decreased final infarct size.<sup>8-11</sup> This cardioprotective effect appeared to be mediated by activation of AMPK and endothelial Nitric Oxide Synthase (NOS).<sup>9</sup> In addition, Yellon's group reported that in diabetic and nondiabetic rat hearts, administration of metformin reduced infarct size.<sup>8, 10, 23</sup> In these studies, the cardioprotective effect was dependent on adenosine receptor stimulation and activation of important signalling molecules of the Reperfusion Injury Salvage Kinase (RISK) pathway. Finally, not only acute single-dose administration of metformin confers cardioprotection, but also chronic administration of metformin limits infarct size<sup>23</sup> and beneficially affects postinfarction myocardial remodelling.<sup>11, 24</sup> Based on these studies, we have recently proposed that metformin treatment is an attractive strategy to limit IR injury in patients suffering a myocardial infarction or patients undergoing cardiac surgery.<sup>7, 25</sup>

To test our hypothesis, we used a well-validated model of IR-induced endothelial dysfunction in the forearm. Endothelial IR-injury is relevant for two reasons. First, myocardial ischemia and reperfusion not only inflicts direct injury to cardiomyocytes, but the endothelial cells are also highly susceptible to IR-injury.<sup>12</sup> Indeed, structural endothelial injury occurs during ischemia and reperfusion, which induces cell swelling and impairment of endothelial-dependent relaxation, which contributes to the so-called 'no-reflow' phenomenon and impedes effective coronary reperfusion.<sup>26, 27</sup> Secondly, endothelial dysfunction is an early sign of cardiovascular disease and is associated with future cardiovascular events.<sup>28-31</sup> Importantly, endothelial dysfunction is associated with a worse outcome in several clinical settings.<sup>32, 33, 34, 35</sup> Thus, IR injury to coronary endothelium could contribute to the increased risk of recurrent atherothrombosis as observed in patients who present with an acute coronary event.

Several previous studies have reported that twenty minutes of forearm ischemia impairs subsequent flow mediated dilation (FMD) of the brachial artery.<sup>13, 14</sup> Indeed, this finding was confirmed in our current study, in which post-IR FMD was 36% lower than baseline FMD. This observation of a lower FMD, even after correction for the potential influence of changes in diameter and the eliciting shear rate stimulus, is in agreement with the concept that IR causes endothelial dysfunction. Subsequent studies demonstrated that this IR-induced endothelial dysfunction can be prevented by ischemic preconditioning, postconditioning, and remote conditioning.<sup>13, 14, 36, 37</sup> Also, statins significantly reduced endothelial IR-injury in this model.<sup>38</sup>

In contrast to the preclinical studies, we did not observe any protective effect of metformin against endothelial IR-injury in our study. There are several potential explanations for this discrepancy. First, murine models of IR-injury might not reflect the human situation. In this regard, many interventions that are promising in animal models do not appear to be effective in clinical trials.<sup>39</sup> In the field of ischemic stroke, only two of approximately 500 neuroprotective strategies that were beneficial in animal models, improved outcome in patients.<sup>39</sup> This translational failure can be due to differences between animal and human (patho)physiology, due to methodological flaws in animal studies, or due to shortcomings of the clinical trial. In the case of metformin-induced cardioprotection, the evidence is rather strong, with cardioprotection shown in mice and rats, with or without diabetes, and with acute as well as chronic administration. The design of our current study is also robust and uses a well-validated model of endothelial IR-injury. Although the study was not blinded, we used a PROBE design, which is a well-accepted design for this kind of studies.

A second explanation could relate to the duration and dose of metformin pretreatment. Interestingly, in several patient groups, including patients with type 1 diabetes and patients with polycystic ovarian syndrome, long-term administration of metformin improves endothelial function, measured with FMD.<sup>40, 41</sup> In our study, however, a three day treatment with metformin did not improve baseline FMD. Animal studies on the cardioprotective effect of metformin, however, have demonstrated that either an acute single dose administration of metformin<sup>9, 10</sup> as well as chronic administration of metformin confer cardioprotection.<sup>23</sup> The dose of metformin used in these preclinical studies was comparable to or even considerably lower than the dose used to treat patients with diabetes in clinical practice.<sup>9, 10</sup> Based on these studies, we used a dose of 3dd500 mg, which is a dose often used to treat patients in clinical practice. Subjects were pretreated for three days to ensure an effective steady-state plasma concentration. The last dose of metformin was taken 3 hours prior to the ischemic episode, which allows for a maximum plasma concentration of metformin at the moment of forearm ischemia. Indeed, the circulating plasma concentration of



metformin immediately before the experiment was comparable to the previous animal studies.

A third potential explanation for the discrepancy between our results and results from previous animal studies is that the mechanism of endothelial IR-injury might differ from IR-injury in cardiomyocytes. However, most strategies that limit myocardial infarct size in animal models also conferred protection against endothelial IR-injury, although some studies could not observe protection.<sup>13, 34, 36, 37</sup>

Fourthly, the findings in the brachial artery may not be representative for the coronary circulation. However, previous studies have reported a good correlation between endothelial responses to flow and vasoactive substances between the brachial and coronary arteries.<sup>42, 43</sup>

In conclusion, we can state that short-term metformin pretreatment does not protect against endothelial dysfunction induced by ischemia-reperfusion in healthy middle aged subject. However, whether these results predict the effect of metformin on myocardial IR-injury and can be extrapolated to subjects with a history of cardiovascular disease or diabetes mellitus is a matter of debate. It is important to realize that many comorbidities, including diabetes mellitus, and comedications can affect the tolerance against ischemia-reperfusion and the efficacy of cardioprotective strategies. Therefore, with interest we await further studies on the effect of metformin on myocardial injury in patients with a myocardial infarction and patients undergoing coronary artery bypass grafting (NCT01217307 and NCT01438723 respectively).

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# 5

## Effect of metformin pretreatment on myocardial injury during coronary artery bypass surgery in patients without diabetes (MetCAB): a double-blind, randomised controlled trial

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## Abstract

**Background:** During coronary artery bypass grafting (CABG), ischemia and reperfusion damages myocardial tissue, and increased postoperative plasma troponin is associated with a worse outcome. We investigated whether metformin pretreatment limits cardiac injury, assessed by troponin concentrations, during CABG surgery in patients without diabetes.

**Methods:** we did a placebo-controlled, double blind, single-centre study in an academic hospital in Nijmegen (Netherlands) in adult patients without diabetes undergoing an elective on-pump CABG procedure. We randomly assigned patients (1:1) in blocks of ten via a computer-generated randomisation sequence to either metformin hydrochloride (500 mg three times a day) or placebo (three times a day) for 3 days before surgery. The last dose was given roughly 3h before surgery. The primary endpoint was the plasma concentration of high-sensitive troponin I at 6, 12, and 24h post-reperfusion after surgery, analysed in the per-protocol population with a mixed-model analysis using all these time points. Secondary endpoints included the occurrence of clinically relevant arrhythmias within 24 hours after reperfusion, the need for inotropic support, time to detubation, duration of stay in the intensive-care unit, and postoperative use of insulin. A per-protocol analysis was performed for all endpoints.

**Findings:** we randomly assigned 111 patients to treatment (57 to metformin and 54 to placebo). Five patients dropped out from the metformin group, and six from the placebo group. 52 patients in the metformin group and 48 patients in the placebo group were included in the per-protocol analysis. Geometric mean high-sensitive troponin I increased from 0  $\mu\text{g/L}$  to 3.67 (95% CI 3.06-4.41) with metformin and to 3.32 (2.75-4.01)  $\mu\text{g/L}$  with placebo at 6 hours after reperfusion; 2.84 (2.37-3.41) and 2.45 (2.02-2.96), respectively, at 12 hours; and to 1.77  $\mu\text{g/L}$  (1.47-2.12) and 1.60  $\mu\text{g/L}$  (1.32-1.94) at 24 hours. The concentration did not differ significantly between the groups (difference 12.3% for all time points [95% CI – 12.4 to 44.1]  $p=0.35$ ). Occurrence of arrhythmias did not differ between groups (three [5.8%] of 52 patients who received metformin vs three [6.3%] of 48 patients who received placebo;  $p=1.00$ ). There was no difference between groups in the need for inotropic support, time to detubation, duration of stay in the intensive-care unit, or postoperative use of insulin. No patients died within 30 days after surgery. Occurrence of gastrointestinal discomfort (mostly diarrhea) was significantly higher with metformin than with placebo (11 [21.2%] of 52 vs two [4.2%] of 48 patients;  $p=0.01$ ).

**Interpretation:** Short-term metformin pretreatment, although safe, does not seem to be an effective strategy to reduce periprocedural myocardial injury in patients without diabetes undergoing CABG surgery.

## Introduction

Coronary artery disease is the leading cause of morbidity and mortality worldwide. For most patients with multiple vessel coronary artery disease, coronary artery bypass graft (CABG) surgery is the preferred strategy for revascularization.<sup>1</sup> However, temporary myocardial ischemia during CABG surgery and the subsequent reperfusion that occurs causes damage to the cardiomyocytes, a marker of which is increased postoperative plasma concentration of troponin. Increased troponin release within the first 24 hours after surgery is independently associated with increased intermediate-term and long-term risk of mortality.<sup>2,3</sup> Therefore, the prognosis after CABG might be improved by strategies that reduce myocardial ischemia-reperfusion injury.<sup>4</sup>

Many preclinical studies report that giving the antihyperglycemic drug metformin either before ischemia occurs or at the moment of reperfusion potently reduces myocardial ischemia-reperfusion injury in the setting of coronary occlusion, both after one dose<sup>5</sup> or when given as a chronic regimen of several doses.<sup>6</sup> Several intracellular signaling cascades contribute to the cardioprotective effect of metformin in animal studies, such as activation of AMP-activated protein kinase (AMPK) and the prosurvival kinase Akt, and an increased intracellular formation of adenosine.<sup>7-9</sup>

Metformin is the most widely used drug in the treatment of type 2 diabetes and is associated with improved cardiovascular prognosis in patients with diabetes compared with other glucose-lowering agents.<sup>10,11</sup> Most guidelines advise to stop metformin before cardiac surgery because of the presumed risk of lactate acidosis.<sup>12</sup> The clinical benefit of this policy, however, is still without evidence. A retrospective analysis in patients with diabetes who underwent CABG even reports that the use of metformin on the day before surgery is associated with less overall morbidity than the use of non-metformin antihyperglycemic agents, although cardiac morbidity and mortality did not differ between treatments.<sup>13</sup>

In this study (MetCAB), we investigated whether short-term pretreatment with metformin minimizes cardiac injury in patients without diabetes undergoing elective CABG surgery. In an accompanying exploratory experiments, we assessed whether treatment with metformin activates key signaling proteins that are involved in cardioprotection.



## Methods

### Study design and participants

We did a randomised, placebo-controlled, double-blind, single-centre trial at the Radboud University Medical Center (Nijmegen, Netherlands). The study protocol was in accordance with the Declaration of Helsinki and was approved by one of the 24 accredited Dutch Medical Research Ethics Committees (CMO Region Arnhem-Nijmegen). This protocol is available online.

We recruited consecutive patients aged at least 18 years accepted for an elective on-pump CABG with or without concomitant valve surgery. After obtaining written informed consent, we screened patients through history taking, physical examination, and laboratory analyses. Exclusion criteria were off-pump surgery, diabetes, renal insufficiency (modification of diet in renal disease glomerular filtration rate <60 ml/min), raised concentrations of liver enzymes (alanine aminotransferase more than three times the upper reference limit), or a documented myocardial infarction within 2 weeks of screening. Additionally, patients taking dipyridamole or xanthine derivatives were excluded because these agents interfere with the endogenous adenosine signaling.

### Randomisation and masking

Patients were randomly assigned (1:1) in blocks of ten to pretreatment with either metformin hydrochloride or placebo by an independent computer-generated randomization sequence (Department of Pharmacy, Radboud University Medical Center, Nijmegen, Netherlands). Patients were enrolled by one of the investigators (SEM). Patients, investigators, trials staff, and the statistician were all masked to treatment allocation, and masking was achieved by over-encapsulation of metformin tablets under Good Manufacturing Practice conditions at the Department of Pharmacy, and the manufacture of matching placebo capsules.

### Procedures

We assigned patients to either metformin hydrochloride (500 mg three times per day) or placebo (three times per day) for 3 days before surgery. In case of postponed surgery, continuation of study drugs was allowed for a maximum of 7 days. Because caffeine is an adenosine receptor antagonist, all patients had to abstain from caffeine consumption for at least 24 hours before surgery. All surgical details are reported in the Appendix.

We measured plasma caffeine and metformin concentrations immediately before surgery. High-sensitive troponin I was measured with the Dimension Vista® system of

Siemens (Newark, USA). The 99<sup>th</sup> percentile of the reference range is 0.045  $\mu\text{g/l}$ , and the 10% coefficient of variation is less than 0.04  $\mu\text{g/l}$ . Additionally, we did AMPD1 genotyping, because variation in this gene is associated with resistance against ischemia-reperfusion injury<sup>14</sup> (analytic details in appendix). We estimated insulin resistance immediately before surgery using the homoeostasis model assessment (HOMA-IR) method, with a HOMA-IR threshold value of at least 2.0 suggesting insulin resistance.<sup>15</sup>

We did two exploratory series of laboratory analyses on the right atrial appendage, which was harvested before the introduction of the extracorporeal circulation in a subgroup of patients (appendix). First, we measured recovery of contractile function after simulated ischemia and reperfusion in the atrial trabeculae, a well established surrogate marker of ischemia-reperfusion injury.<sup>16</sup> Additionally, we measured phosphorylation of AMP-activated protein kinase and Akt and translocation of hexokinase II in atrial trabeculae with western blotting.

## Outcomes

Postoperative high-sensitive plasma troponin I concentration was the primary endpoint, measured before surgery and at 6, 12, and 24 h after release of the aortic clamp (after surgery). Values at all time points were used to calculate the primary endpoint.

Secondary endpoints were the occurrence of clinically relevant arrhythmias during the first 24 h after reperfusion (retrieved from medical charts), the need for inotropic support, time to detubation, duration of stay in the intensive-care unit, use of exogenous insulin, recovery of contractile function of isolated atrial trabeculae after ex vivo-simulated ischemia-reperfusion, and expression and activation of AMP-activated protein kinase, Akt, and hexokinase II in isolated atrial trabeculae. We did not measure renal ischemia-reperfusion injury because of logistic reasons.

For safety monitoring, plasma creatinine (to assess renal function), pH, and lactate concentrations were measured at 6, 12, and 24 h after surgery. An independent data safety monitoring board did a prespecified safety analysis after 50% of patients were enrolled. This analysis did not reveal any concerns with respect to safety.

## Statistical analysis

We based the size calculation on the plasma troponin I concentrations at 12 hours after CABG in 880 patients from our own clinic in the period 2006-2008 (6.76  $\mu\text{g/L}$  [SD 12.96]). The mean plasma troponin I concentration after <sup>10</sup>log transformation was 0.53 [SD 0.41], which we used in our sample size calculation. We calculated a total

sample size of 80 patients would provide a power of 90% with an  $\alpha$  of 0.05 to detect a 50% reduction in postoperative troponin I release (i.e. a reduction from 6.76  $\mu\text{g/L}$  to 3.38  $\mu\text{g/L}$ , which equals a reduction in the  $^{10}\log$  transformed troponin I concentration from 0.53  $\mu\text{g/L}$  to 0.23  $\mu\text{g/L}$ ). A reduction of 50% was expected on the basis of preclinical studies on the infarct size-limiting effect of metformin<sup>7,9</sup> and on the effect of remote ischemic preconditioning on release of troponin I after CABG surgery.<sup>14</sup> To enable analysis of the primary endpoint in patients with the CC genotype of *AMPD1*, which is present in 80% of the white population,<sup>17</sup> we added 20% to the calculated 80 patients, and therefore aimed to include 100 assessable patients in our study.

Plasma high-sensitive troponin I was not normally distributed, and results are therefore presented as geometric means with 95% CI. We used a log transformation to correct for these skewed data and a mixed-model analysis to assess differences in postoperative plasma high-sensitive troponin I to accommodate the repeated measurements by incorporating a participant-dependent intercept; the independent variables in this model were time after reperfusion and treatment group. We assessed possible differences between the two treatment groups over time by incorporating a time-by-treatment interaction. We did a covariate analysis to study the effect of propofol anesthesia on the effect of metformin. A two-sided *P*-value of less than 0.05 was regarded as significant. Continuous secondary endpoints that were normally distributed are presented as mean (SD) and analyzed using an unpaired student's *t*-test. Discrete secondary endpoints were analyzed using a Chi-Square analysis or a Fisher's exact test in case of few events. Continuous data that were not normally distributed are presented as medians (IQR) and were analyzed using a Mann-Whitney *U* test. We calculated correlations between the plasma metformin concentration and the duration of ischemia and the primary endpoint with a Spearman rank order correlation. Because our study is a *proof-of-concept* study, we did a per-protocol analysis (we defined the per-protocol population as all patients who completed the protocol; i.e. all patients minus the drop-outs). Additionally, we did an intention-to-treat analysis of the primary endpoint.

For the laboratory experiments on the effect of simulated ischemia-reperfusion on contraction of atrial trabeculae, all data are presented as mean (SD). For each contraction of the trabeculae, we calculated developed force (difference between maximal tension during contraction and the minimum tension during relaxation). This variable was averaged for the last 5 minutes during equilibration and each subsequent 5 minute period. Data are expressed as percentage of baseline developed force. A paired student's *t* test was used to compare in-vitro treatment of trabeculae with control versus metformin during the last 10 minutes of final reperfusion. To assess the

effect of in-vivo treatment with metformin, an unpaired student's *t* test was used to compare vehicle-treated trabeculae of both groups.

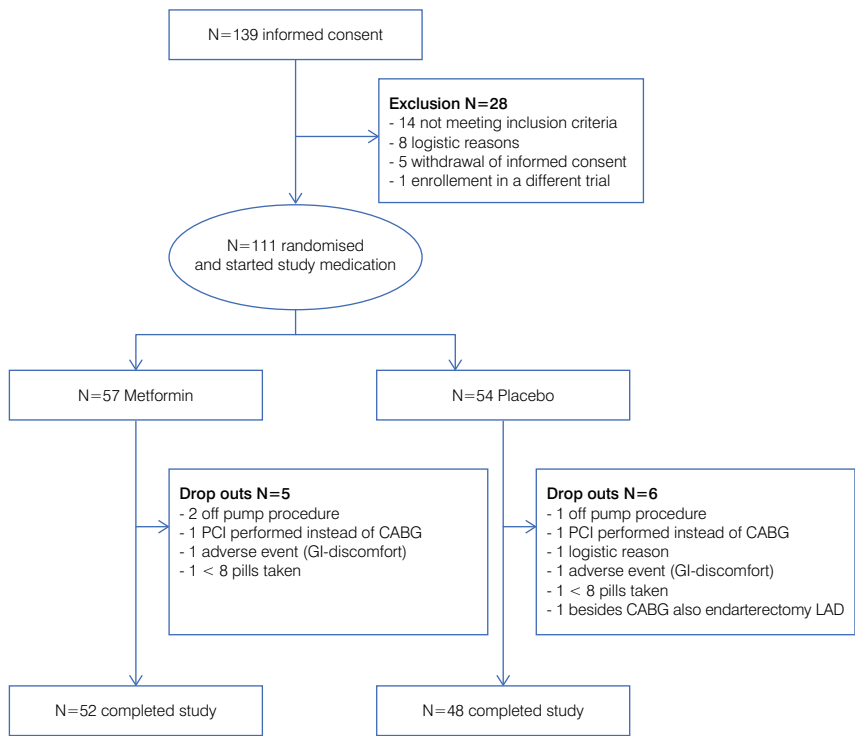
All Western blot data was normalized to placebo (dividing all individual values by the mean of the placebo group) and presented as mean (SE). We used an unpaired student's *t* test was used to compare the metformin and placebo groups. SAS version 9.2 was used for all statistical analyses. This study is registered with ClinicalTrials.gov, number NCT01438723.

## Results

Between Nov 8, 2011, and Nov 22, 2013, 139 consecutive patients provided informed consent. 28 patients were ineligible (appendix), therefore we randomly assigned 111 patients to treatment (57 to metformin and 54 to placebo). Five patients dropped out from the metformin group, and six from the placebo group. 52 patients in the metformin group and 48 patients in the placebo group were included in the per-protocol analysis (figure1). Baseline characteristics of the per-protocol population did not differ between the two groups (table 1). Surgical details are described in the appendix.

Immediately before surgery, mean plasma metformin concentrations were 8.42  $\mu\text{mol/L}$  (SD 5.98) for the metformin pretreated group (range 1.16-24.91  $\mu\text{mol/L}$ ) and 0.0  $\mu\text{mol/L}$  (0.0) for the placebo group. Caffeine plasma concentrations were less than 5.2  $\mu\text{mol/L}$  in 91 patients; nine had a concentration of more than 5.2  $\mu\text{mol/L}$  (range 5.2-24.2  $\mu\text{mol/L}$ ). Analysis of the primary endpoint did not change after exclusion of these patients (data not shown). On the day of surgery plasma fasting glucose concentrations did not differ between groups (metformin group mean 5.3 mmol/L [SD 0.9], placebo group 5.2 [SD 2.2],  $p=0.35$ ), nor did pre-operative plasma fasting insulin concentration (metformin group 106.26 pmol/L [75.7], placebo 114.6 pmol/L [86.12],  $p=0.59$ ). HOMA-IR was also similar in both groups (metformin 4.8 [SD 8.6], placebo 4.5 [SD 4.6],  $p=0.24$ ) and showed insulin resistance in our study population.

None of the patients died within the first 30 days after surgery. Baseline concentrations of hs-troponin I were below the detection limit of 0.02  $\mu\text{g/L}$  in the 79 patients. In 21 patients baseline hs-troponin levels were more than 0.02  $\mu\text{g/L}$  (11 [21.2%] of 52 patients in the metformin group and 10 [20.8%] of 48 patients in the placebo group ( $P=0.97$ , Pearson's chi square test), with a range of 0.21-1.88  $\mu\text{g/L}$  in the metformin group and 0.03-0.49  $\mu\text{g/L}$  in the placebo group ( $P=0.51$ , Mann Whitney *U* test).



**Figure 1** Trial profile.

CABG= coronary artery bypass grafting. GI= gastrointestinal. LAD= left anterior descending artery. PCI= percutaneous coronary intervention.

Geometric mean high-sensitive troponin I increased from  $0 \mu\text{g/L}$  to  $3.67 \mu\text{g/L}$  (95% CI 3.06-4.41) with metformin and to  $3.32 \mu\text{g/L}$  (2.75-4.01) with placebo at 6 h after reperfusion; to  $2.84 \mu\text{g/L}$  (2.37-3.41) with metformin and  $2.45 \mu\text{g/L}$  (2.02-2.96) with placebo at 12 h; and to  $1.77 \mu\text{g/L}$  (1.47-2.12) with metformin and  $1.60 \mu\text{g/L}$  (1.32-1.94) with placebo at 24 h. We noted no significant interaction between time and treatment group ( $p = 0.73$ ), so the interaction was dropped from the model. The concentrations of high-sensitive troponin I did not differ between the groups at 6, 12, and 24 h after reperfusion (difference 12.3% [95% CI: -12.4% to 44.1],  $p = 0.35$ ; figure 2). An intention-to-treat analysis showed similar results: difference 15.2% (-11.1 to 41.5,  $p = 0.26$ ). In the metformin group, metformin concentrations did not correlate with high-sensitive troponin I concentrations ( $r = -0.14$ ,  $p = 0.33$ ). With regard to the use of propofol

**Table 1** Baseline characteristics.

	Metformin (N=52)	Placebo (N=48)
Age	65.2 (8.8)	64.5 (9.8)
Male	43 (83)	38 (79)
Body mass index, kg/m <sup>2</sup>	27.5 (4.1)	27.7 (3.5)
Blood pressure, mmHg		
Systolic	147 (23)	147 (22)
Diastolic	82 (11)	83 (11)
Heart rate, beats/min	65 (9)	64 (11)
Heart rhythm at screening	49 (94.2)	47 (97.9)
Sinus rhythm	3 (5.8)	1 (2.1)
Atrial fibrillation	10 (19)	11 (22)
<b>Concomitant valve surgery</b>		
Aortic valve	9 (17)	9 (19)
Mitral valve	1 (2)	1 (2)
Combination aortic/mitral	0 (0)	1 (2)
<b>Cardiovascular related history</b>		
Angina pectoris CSS class		
I	13 (25%)	10 (21%)
II	18 (35%)	17 (35%)
III	16 (31%)	16 (33%)
IV	5 (10%)	5 (10%)
Previous myocardial infarction	20 (38.5%)	12 (25%)
Previous CABG	3 (6%)	0 (0%)
Previous PCI	13 (25%)	13 (27%)
Left ventricular ejection fraction at screening		
> 55%	21 (40%)	17 (35%)
35-55	9 (17%)	11 (23%)
< 35%	1 (2%)	2 (4%)
Unknown	21 (40%)	18 (38%)
<b>Cardiovascular risk factors*</b>		
Hypertension	30 (58%)	30 (63%)
Smoking	15 (29%)	14 (29%)
Dyslipidemia	19 (37%)	15 (31%)
Positive family history (first degree, < 60 y)	25 (48%)	28 (58%)

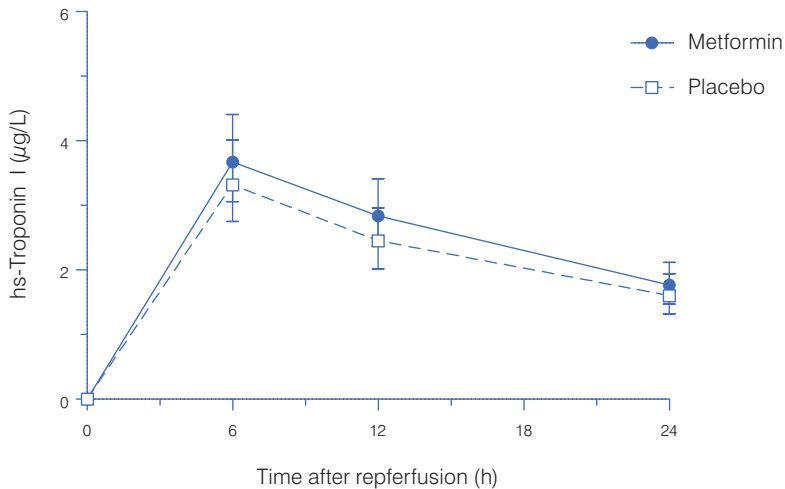
**Table 1** Continued.

	<b>Metformin (N=52)</b>	<b>Placebo (N=48)</b>
<b>Co-medication</b>		
Aspirin	45 (87%)	40 (83%)
$\beta$ -blocker	43 (83%)	40 (83%)
Statin	40 (77%)	40 (83%)
ACE- inhibitor	21 (40%)	17 (35%)
AT2 receptor antagonist	8 (15%)	10 (21%)
Calcium channel blocker	10 (19%)	9 (19%)
Nitrates	34 (65%)	29 (60%)
Diuretic (loop and thiazide)	15 (29%)	15 (31%)
Clopidogrel	14 (27%)	6 (13%)
<b>Laboratory values at screening</b>		
Glucose, mmol/L	6.2 (1.7)	6.4 (1.6)
Total cholesterol, mmol/L	4.7 (0.99)	4.7 (1.3)
Creatinine, mmol/L	81.9 (11.9)	81.9 (12.0)
GFR (MDRD) ml/min/1.73 m <sup>2</sup>	77.3 (9.3)	77.1 (9.7)
<b>AMPD genotype</b>		
CC	34 (65%)	33 (69%)
CT	18 (35%)	13 (27%)
TT	0 (0%)	2 (4%)
Number of investigational pills taken, median (IQR)	9 (9-10)	9 (9-10)

Data are number (%), mean (SD), or median (IQR). CSS= Canadian Cardiovascular Association. CABG= coronary artery bypass grafting. PCI= percutaneous coronary intervention. ACE= angiotensin-converting enzyme. GFR= glomerular filtration rate. MDRD= modification of diet in renal disease formula. \* according to the treating physician as noted in the medical files.

treatment (15 [29%] of 52 patients in the metformin group vs 19 [40%] of 48 in the control group), propofol treatment did not modulate the effect of metformin on high-sensitive troponin I concentration: in a covariate analysis, propofol had no effect ( $P=0.47$ ), and we did not find a significant interaction with the treatment ( $P=0.98$ ).

The *AMPD1* 34C>T CT or TT genotype was present in 18 (35%) of 52 patients in the metformin group and in 15 (31%) of 48 patients in the placebo group. No significant interaction between the genotype and the effect of metformin on high-sensitive troponin I release was noted (data not shown).



**Figure 2** Post-operative hs-troponin I concentration in plasma.

(geometric mean with 95% confidence interval). Solid line: metformin (N=52), dashed line: placebo (N=48). The two curves do not differ significantly (Mixed Model analysis after log transformation,  $p = 0.35$ ).

The duration of ischemia (mean 68 minutes [SD 23; range 24-126]), was correlated with high-sensitive troponin I concentrations ( $r=0.41$ ,  $p<0.0001$ ).

The occurrence of arrhythmia's within 24 hours after surgery, including atrial fibrillation or atrial tachycardia did not differ between groups (three [5.8%] of 52 patients who received metformin vs three [6.3%] of 48 patients who received placebo;  $p=1.00$ ). Metformin did not affect time to detubation, length of stay at in an intensive care unit, or need for inotropic support. There was no difference between groups in the number of patients who received insulin treatment after surgery (median 18.9 units [IQR 7.2-31.8] in 85% of patients in the metformin group vs median 19.7 units [11.1-29.0]) in 85% of patients in the control group; table 2).

No differences were noted in post-operative changes in kidney function or pH levels between groups. Median creatinine concentrations at 6, 12, and 24 h after surgery were 86  $\mu\text{mol/L}$  (IQR 74-97), 87  $\mu\text{mol/L}$  (74-96), and 79  $\mu\text{mol/L}$  (69-88), respectively, in the metformin group, versus 83  $\mu\text{mol/L}$  (74-99), 79  $\mu\text{mol/L}$  (68-94), and 73  $\mu\text{mol/L}$  (61-91), respectively, in the placebo group ( $p=0.22$ ). In the metformin group, median



**Table 2** Secondary endpoints.

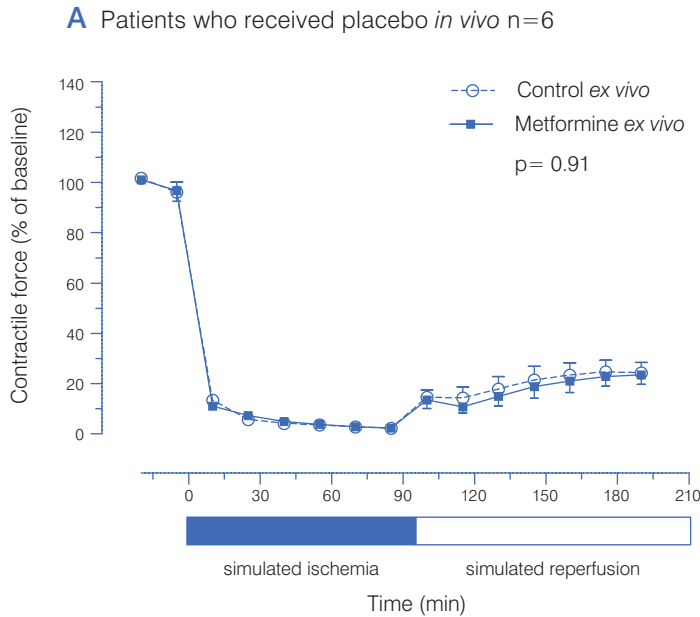
	Metformin (N=52)	Placebo (N=48)	p-value
Days on ICU, median (IQ range)	1 (1-1)	1 (1-1)	0.78
Number of patients on inotropy	40 (77%)	31 (64%)	0.08
Duration of inotropic support, hours, median (IQ range)	15 (7.25-20)	14 (0-21)	0.74
Time to detubation, hours, median (IQ range)	12 (10-17.8)	12 (11-14.8)	0.71
Clinical relevant arrhythmias < 24 hours			
None	49 (94.2%)	45 (93.7%)	1.0
AF/SVT de novo	2 (3.8%)	0 (0%)	0.50
Sinus bradycardia in need for pacemaker	1 (1.9%)	0 (0%)	1.00
AV conduction disturbance	0 (0%)	0 (0%)	-
VT/VF	0 (0%)	2 (4.2%)	0.23
Other	0 (0%)	1 (2.1%)	0.48

ICU= intensive care unit. AF= atrial fibrillation. SVT= supraventricular tachycardia. AV= atrioventricular. VT= ventricular tachycardia. VF= ventricular fibrillation.

lactate concentrations at 6, 12 and 24 hours after surgery were 1.4 (IQR 1.2-2.0), 1.8 mmol/L (1.3-2.6) and 1.8 mmol/L (1.2-2.3), respectively, versus 1.2 mmol/L (1.0-2.2) mmol/L, 2.0 mmol/L (1.5-2.7) and 1.9 mmol/L (1.4-2.7) in the placebo group,  $p=0.21$ ). Bleeding complications or need for additional surgical intervention did not differ between the groups. Common adverse events included the occurrence of gastrointestinal discomfort (mostly mild and self-limiting diarrhea), which was higher in the metformin group than in the placebo group (11 [21.2%] of 52 vs two [4.2%] of 48 patients; see appendix for other details about other adverse events).

Post-ischemic recovery of contractile function was determined in atrial trabeculae of 16 patients (ten receiving metformin, six receiving placebo). The baseline characteristics of the patients in whom these studies were undertaken are summarized in the appendix. Baseline contractile force was similar for all groups. Simulated ischemia reduced contractile force to a similar extent in trabeculae of patients pretreated with metformin or placebo before surgery. Post-ischemic recovery of contractile function did not differ between the control trabeculae (i.e. the trabeculae treated with vehicle in vitro) of the patients treated before surgery with metformin or placebo (30% [SD 14] vs 24% [10],  $p=0.38$ ; figure 3). Irrespective of treatment before surgery, additional metformin added ex vivo did not enhance recovery of contractile function after ischemia-reperfusion (figure 3). Atrial tissue of 22 patients (12 in the placebo group, ten in

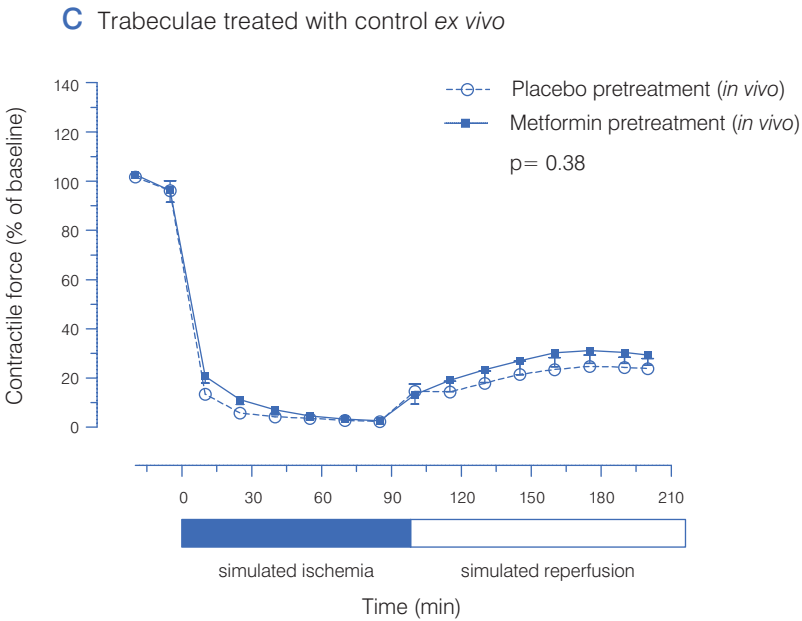
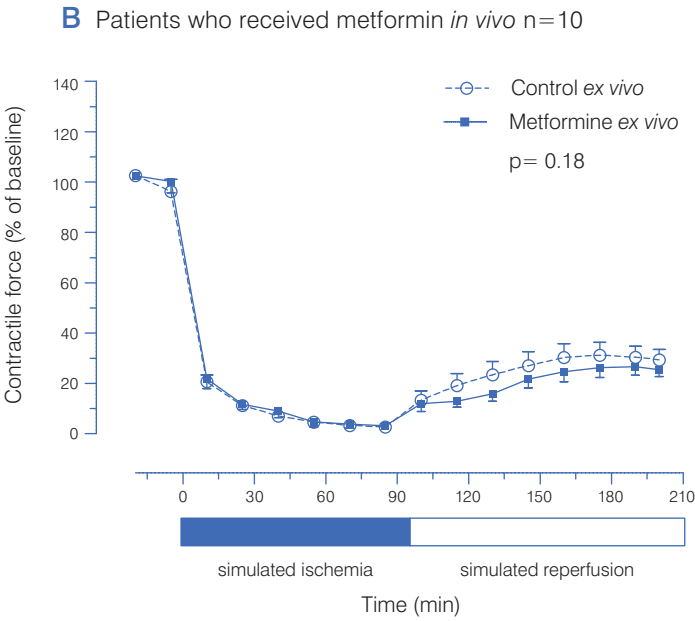
the metformin group) was analyzed for phosphorylated AMP-activated protein kinase, phosphorylated Akt, and hexokinase II content by western blot (appendix). Compared with control, metformin treatment increased phosphorylated AMP-activated protein



**Figure 3** The course of contractile force (expressed as percentage of baseline force) in time.

Data are mean (SD). Contractile force from trabeculae harvested during surgery from six patients randomly assigned to pretreatment with placebo (**A**) and ten patients randomly assigned to pretreatment with metformin (**B**); one trabecula from each patient was treated ex vivo with metformin and the other was treated ex vivo with control (dimethyl sulfoxide). (**C**) Comparison of trabeculae treated with vehicle ex vivo from patients pretreated with metformin versus placebo before surgery.

kinase by a mean of 10% (SE 3;  $p=0.03$ ), without an effect on total AMP-activated protein kinase. Additionally, phosphorylated Akt was increased by a mean of 28% (SE 7;  $p=0.005$ ). We noted no effect of metformin on total Akt, total hexokinase II, or mitochondrial-bound hexokinase II (appendix).



**Figure 3** Continued.

## Discussion

In this study, metformin did not limit myocardial ischemia-reperfusion injury, detected with plasma high-sensitive troponin I concentration, in patients without diabetes undergoing CABG surgery. Additionally, metformin did not affect any of the secondary clinical endpoints. In accordance with these clinical endpoints, metformin did not improve the *ex vivo* post-ischemic recovery of contractile function in isolated atrial trabeculae, a well-established surrogate endpoint for ischemia reperfusion injury.

The search for direct cardioprotective effects of metformin has been fueled by the findings that patients with type 2 diabetes given metformin have a better cardiovascular outcome than patients given other glucose-lowering agents, despite similar glycaemic control.<sup>10,11</sup> Additionally, many preclinical studies report beneficial effects of metformin on atherosclerosis development, cardiac remodelling, and myocardial ischemia-reperfusion injury. In murine models of myocardial infarction, giving metformin, either before ischemia or at reperfusion, consistently minimizes infarct size.<sup>5</sup> Data from human beings that show a cardioprotective effect of metformin are scarce. In a retrospective analysis<sup>18</sup>, metformin-treated patients with diabetes who had a myocardial infarction seemed to have lower plasma troponin T concentrations than patients given other antidiabetic drugs, but this study design is prone to selection bias and confounding. In a randomised prospective pilot study<sup>19</sup> in patients with metabolic syndrome, 1 week of treatment with metformin reduced plasma high-sensitive troponin I concentrations after elective percutaneous coronary intervention. This study, however, had an open-label design and only a minimum amount of cardiac ischemia, as substantiated by the very low troponin I concentrations after percutaneous coronary intervention. By contrast, metformin did not reduce endothelial ischemia-reperfusion injury in healthy people measured with flow-mediated dilation after induction of forearm ischemia by upper-arm cuff inflation.<sup>20</sup> Our finding that the cardioprotective effects of metformin in preclinical studies do not translate into a reduction in troponin I concentration after CABG are in line with findings from other randomised controlled trials<sup>21,22</sup> that showed that metformin does not limit atherosclerosis or postinfarct remodelling in patients. In patients with established coronary heart disease but without diabetes, long-term treatment with metformin tended to increase the concentration of circulating high-sensitive troponin T at 6, 12, and 18 months of treatment to a mean of 1.0 pg/mL (based on all time points), 95% CI 0.0-1.9;  $P=0.05$ .<sup>21</sup> These findings raise the question of why the beneficial effects of metformin on cardiac ischemia-reperfusion injury in animal models do not translate to the clinical setting. First, post-operative troponin concentration might not be an accurate marker of myocardial ischemia-reperfusion injury. However, we noted a significant association between duration of myocardial ischemia and troponin I concentration. Moreover, many previous studies report that

interventions that specifically target ischemia-reperfusion injury reduce the concentration of troponin I after CABG surgery.<sup>4,14</sup> Second, the duration or dose, or both of metformin in clinical trials might be insufficient to activate prosurvival pathways in cardiac tissue. However, in our study, ex vivo experiments show that metformin pretreatment did activate pivotal prosurvival proteins. Additionally, a much higher dose of metformin did not affect ischemia-reperfusion injury in isolated atrial trabeculae. In murine models of myocardial infarction, substantially lower doses of metformin induced profound cardioprotection, although plasma concentrations were not reported in these studies.<sup>7</sup> With regard to the duration, both one dose and a chronic 4-week regimen of metformin reduces infarct size in rodents.<sup>6,7,9</sup> Thus, it is unlikely that a longer duration of metformin pretreatment would have resulted in a clinically relevant protection. Third, a general explanation for absence of translation is that animal studies are predominantly done in young and otherwise healthy animals. By contrast, human patients with cardiovascular disease are older, and have comorbidities and co-medication, which can all interfere with cardioprotection.<sup>23</sup> Among other factors, older age and insulin resistance, which were present in our patients, are associated with an impaired cardioprotective effect of preconditioning, and drugs such as statins (used in most of the patients in our study) offer cardioprotective effects themselves.<sup>23-25</sup> Propofol anesthesia can disrupt protection by remote ischemic preconditioning,<sup>4</sup> but did not modulate the effect of metformin in our study. Additionally, publication bias might exist in preclinical studies resulting in selective publication of positive studies.

Animal studies have identified several signaling cascades that contribute to metformin-induced cardioprotection, including activation of myocardial AMP-activated protein kinase and Akt.<sup>7,8</sup> Also, increased intracellular formation of adenosine and subsequent adenosine receptor stimulation contribute to the direct cardioprotective effect of metformin.<sup>9</sup> Finally, mitochondrial hexokinase II translocation increases resistance against ischemia-reperfusion injury, and metformin can induce mitochondrial hexokinase II translocation.<sup>26</sup> In ex-vivo analyses of tissue from a subset of patients, we noted activation of AMP-activated protein kinase and Akt in atrial tissue, but to a smaller extent than in murine models.<sup>7,8</sup> By contrast, we did not find hexokinase II translocation. Notably, cardioprotective signaling pathways show interspecies differences – e.g, Akt activation has been reported to be less important in ischemic post-conditioning in larger mammals than in rodents.<sup>27</sup> We conclude that the mild activation of AMP-activated protein kinase and Akt by metformin might not have been sufficient to offer significant cardioprotection.

AMPD is a key intracellular enzyme catalyzing the conversion of AMP into inosine monophosphate (IMP). The common loss-of-function variant 34C>T is associated

with an impaired conversion of AMP into IMP, which facilitates the formation of adenosine, and is associated with increased resistance against ischemia-reperfusion injury and better outcome in patients with cardiovascular disease.<sup>17,28</sup> We postulated that in patients with the variant allele, the metformin-induced increase in adenosine formation would be augmented, and we did a prespecified subgroup analysis for the primary endpoint in relation to this variant. This analysis, however, did not support our hypothesis; although our study was powered to detect an effect of metformin in the patients with the CC genotype, it was not powered to detect a larger effect of metformin in patients with the CT/TT genotype versus patients with the CC genotype.

Current practice is to temporarily stop giving metformin before CABG surgery because of the presumed increased risk of lactic acidosis. From the scientific literature, however, evidence does not support this assumption, especially for patients with normal kidney function.<sup>29</sup> In our study, giving metformin to patients with preserved renal function did not result in higher lactate concentration, a higher occurrence of lactate acidosis, or the occurrence of any serious adverse event.

Our study has some limitations. First, it was not powered to detect differences in clinical endpoints. However, the plasma troponin I concentration within the first 24 hours after surgery is a well-established surrogate marker of ischemia-reperfusion injury, which is independently associated with prognosis. Second, the clinical secondary endpoints, such as length of stay in the intensive-care unit, might be affected by factors other than the clinical condition of the patient (e.g. logistic reasons), but this is unlikely to have affected our results because of the block-randomised, double-blind design. Third, we only measured plasma troponin I and creatinine for 24 hours after reperfusion. For troponin I, a longer follow-up would not have improved the statistical power because troponin I was already low at 24 hours. For creatinine, however, we might have missed the occurrence of acute kidney injury, because most CABG-related acute kidney injury is detected only on day 2 after surgery.<sup>30</sup> Fourth, the exploratory laboratory analyses were done, for practical reasons, on right atrial tissue, but effects might differ from the left ventricular tissue. Together with the results of randomized controlled trials focusing on atherosclerosis and post-infarct remodeling,<sup>21,22</sup> results from our study show that the beneficial effects of metformin on atherosclerosis, myocardial ischemia-reperfusion injury and post-infarction remodelling that were noted in preclinical studies do not translate into a clinical benefit, at least in patients without diabetes. Thus, the improved cardiovascular prognosis that has previously been reported in patients with diabetes given metformin is likely to be due to effects on glucose metabolism and body weight, rather than due to direct cardiovascular protective effects.

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# 6

## Effect of dipyridamole on myocardial reperfusion injury: A double-blind randomized controlled trial in patients undergoing elective coronary artery bypass surgery.

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## Abstract

**Background:** Dipyridamole reduces reperfusion-injury in preclinical trials and may be beneficial in patients undergoing coronary angioplasty, but its effect in patients undergoing coronary artery bypass grafting (CABG) is unknown. We hypothesized that dipyridamole limits myocardial reperfusion-injury in patients undergoing CABG.

**Trial design:** Double-blind trial randomizing between pretreatment with dipyridamole or placebo.

**Methods and Results:** 94 patients undergoing elective on-pump CABG were recruited between February 2010 and June 2012. The primary endpoint was plasma high-sensitive (hs-) troponin-I at 6, 12 and 24 hours after reperfusion. Secondary endpoints were the occurrence of bleeding, arrhythmias, need for inotropic support and intensive care unit length of stay. Finally, 79 patients (33 dipyridamole) were included in the per-protocol analysis. Dipyridamole did not significantly affect post-operative hs-troponin-I (change in plasma hs-troponin I -3% (95% CI from -23% to 36%);  $p > 0.1$ ). Secondary endpoints did not differ between groups.

**Conclusion:** Dipyridamole prior to CABG does not significantly reduce post-operative hs-troponin release.

## Introduction

During coronary artery bypass grafting (CABG), myocardial ischemia and reperfusion (IR-) injury contributes to postoperative appearance of circulating troponin. Importantly, postoperative troponin levels are associated with worse outcome.<sup>1, 2</sup> Therefore, much effort is put into developing novel strategies to limit peri-procedural myocardial IR-injury, such as ischemic preconditioning.<sup>3, 4</sup> Preclinical studies have shown that the endogenous nucleoside adenosine is a crucial mediator of ischemic preconditioning.<sup>5, 6</sup> Development of pharmacological strategies to mimic or to enhance cardioprotection by ischemic preconditioning might provide us with tools to induce a state of organ protection against IR-injury in patients at risk for acute ischemic events.

Studies in which adenosine is administered to patients with a myocardial infarction have shown disappointing results, most likely due to the very short half-life of adenosine and the active metabolic barrier of the endothelium, preventing circulating adenosine from reaching myocardial cells.<sup>7, 8</sup> Dipyridamole augments the endogenous interstitial adenosine concentration by inhibiting the equilibrative nucleoside transporter (ENT) transporter.<sup>9-11</sup> In preclinical animals studies<sup>12-15</sup>, and studies in healthy volunteers and patients undergoing coronary angioplasty (PCI), dipyridamole limits IR-injury.<sup>11, 16-18</sup> Protection by dipyridamole against peri-procedural cardiac injury in the setting of CABG has never been addressed. Therefore, this study was designed to test the hypothesis that oral pre-treatment with dipyridamole prevents IR-injury in patients who undergo elective CABG. We also studied whether the cardioprotective effect of dipyridamole was dependent on the genotype encoding for adenosine monophosphate deaminase (AMPD), which is a central enzyme in the intracellular metabolism of adenosine.<sup>19</sup>

## Methods

### Patients

We recruited consecutive adult patients accepted for elective on-pump CABG between February 2010 and June 2012 at the Radboud university medical center. We excluded patients who suffered from myocardial infarction within two weeks before screening and those with asthma, since dipyridamole could aggravate this pulmonary condition.<sup>20</sup> Additionally, patients taking xanthine derivatives (such as theophylline), sulphonylurea derivatives, insulin or metformin were excluded since these agents interfere with the endogenous adenosine metabolism.<sup>21-23</sup> To minimize an additive risk of bleeding we also excluded patients taking oral anticoagulants or antiplatelet drugs other than aspirin within 8 days before surgery. As caffeine is an effective

adenosine receptor antagonist, all patients had to abstain from caffeine consumption for at least 24 hours before surgery.<sup>24</sup> Compliance to this restriction was controlled by measuring plasma caffeine concentration on the day of surgery. This study has been approved by the local ethics committee and is therefore been performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

Preparation, conduct and analysis of this trial complied to Good Clinical Practice guidelines. This trial was prospectively registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01295567).

### Pharmacological intervention and blinding

Patients randomly received either dipyridamole slow release (200 mg b.i.d.) or placebo (b.i.d.) for 3 days prior to surgery. The dose of 200 mg b.i.d. was selected because we have previously shown that this dose effectively blocks *ex vivo* nucleoside uptake in red blood cells in humans and significantly limits IR-injury in a forearm model of IR-injury.<sup>11</sup> In case of postponed surgery, continuation of study medication was allowed for a maximum of 7 days, otherwise that patient was excluded. Compliance with this therapy was controlled by pill count and by measuring the plasma dipyridamole concentration on the day of surgery. To guarantee blinding of patients and investigators, dipyridamole was over-encapsulated under Good Manufacturing Practice (GMP) conditions at the department of Clinical Pharmacy of the Radboud University Medical Center. An independent Data Safety Monitoring Board performed a prespecified safety analysis after inclusion of 50 patients. This analysis did not reveal any concerns with respect to safety.

### CABG procedure

Anesthesia was performed with sufentanil, midazolam, rocuronium and propofol according to clinical judgment. As sufentanil and propofol may influence IR-injury we recorded the administration of these substances and for sufentanil we also recorded the cumulative dose used.<sup>25</sup> Only patients who underwent on-pump surgery were included in our efficacy analysis since only in these patients cardioplegia with global cardiac ischemia is applied. All patients were operated using standard cardiopulmonary bypass technique, aortic and right atrial (two stage) cannulation, with membrane oxygenator, moderate hemodilution and mild systemic hypothermia (32-34°C). Conventional myocardial protection was performed by intermittent infusion of cold 4°C cardioplegic solution. The choice of the cardioplegic solution was left to the distinction of the surgeon. Distal anastomoses were performed during a single period of cross-clamping, proximal anastomoses using a partial occluding clamp. Before weaning the patient off extracorporeal circulation, rewarming was initiated to a rectal

temperature of 35°C and the hematocrit was increased to a minimum value of 0.24 l/l. Surgical risk estimation was performed using the logistic EuroSCORE (see table 1).<sup>26</sup>

### Outcome parameters

Plasma high-sensitive (hs-) troponin I was the primary endpoint of this trial and was determined before surgery and 6, 12, 24 h after reperfusion (defined as release of aortic clamp). Hs-troponin I concentrations were analysed on the Dimension Vista® System (Siemens®, Healthcare Diagnostic, The Hague, The Netherlands) at the department of Clinical Chemistry (KCH Laboratory), Jeroen Bosch Hospital, Den Bosch, The Netherlands. The method used is a homogeneous, sandwich chemiluminescent immunoassay based LOCI® technology. The lower limit of detection is 0.02 µg/L.

We also collected the following clinical data prospectively (secondary endpoints): the occurrence of arrhythmias during the first 24 hours after reperfusion (as documented in clinical chart), the need for prolonged inotropic support (>24 hours), prolonged Intensive Care Unit (ICU) stay (>48 hours), clinically relevant bleeding complications (defined as bleeding complications that required additional surgical intervention and/or prolonged hospital stay) and cumulative volume of blood collected from chest tubes post-surgery. All adverse events that occurred during the treatment period and first 48 hours after surgery were documented according to Good Clinical Practice.

Plasma Lipoprotein-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>) was determined with the ELISA kit from USCN Life Science Inc. (Wuhan, People's Republic of China). Interleukin-6 (IL-6) was measured with the ELISA kit from Sanquin (Pelipair; M9316). High-sensitive CRP (hs-CRP) was determined using the BNII Behring Nephelometer Analyser from Siemens. Matrix metalloproteinase 9 (MMP-9) was analysed using the ELISA kit from Invitrogen (Camarillo, CA). Monocyte chemotactic protein 1 (MCP-1) was measured using the ELISA kit from BD Bioscience (San Diego, CA). Pentraxin-related protein 3 (PTX-3) was determined with the ELISA kit from Adipo Bioscience (Santa Clara, CA) according to the manufacturer's instructions. Adiponectin was measured using the ELISA kit from R&D (Human adiponectin DuoSet; DY1065). All these plasma markers for inflammation were determined before start and after completion of study medication (just before surgery).

Plasma caffeine concentrations were determined by use of reversed-phase HPLC with ultraviolet detection set at 273 as described previously.<sup>27</sup> Dipyridamole concentrations were determined with the use of LC-MS/MS quantification, using an Accela U-HPLC (Thermo Fischer Scientific) coupled to a TSQ Vantage (Thermo Fisher Scientific) triple quadrupole mass spectrometer. The compounds were separated on a Grace

Vision HT C18 column (50x2.0 mm, 1.5  $\mu$ m particle size). As internal standard we used dipyridamole-D20. Solvent A consisted of 5 mM NH<sub>4</sub>, Solvent B consisted of Methanol. Flow rate was 200  $\mu$ l/min. Detection of dipyridamole and the internal standard was based on isolation of the protonated molecular ion, [M + H]<sup>+</sup> and subsequent MS/MS fragmentations and a selected reaction monitoring (SRM) were carried out. For dipyridamole the parent was 505.3 m/z and products were 385.4 and 429.4 m/z. For the internal standard the parent was 525.4 m/z and products were 405.5 and 449.5 m/z.

For *AMPD1*-genotyping, blood was stored at -80 °C until DNA isolation. Genomic DNA isolation was performed using a standard desalting protocol.<sup>28</sup> Genotyping of the genetic variant 34C>T (rs17602729) in the *AMPD1* gene was performed by Taqman analysis (assay\_ID C\_\_33603912\_10) according to the protocol of the manufacturer (Life Technologies Europe, Bleiswijk, The Netherlands).

### Statistical analysis

Plasma hs-troponin I was not normally distributed and is presented as median with interquartile range. A log transformation was used to correct for these skewed data and a mixed model analysis was used to assess difference in post-operative plasma hs-troponin I in order to accommodate the repeated measurements by incorporating a subject dependent intercept. A value of  $p < 0.05$  was regarded as significant (SAS version 9.2, Cary, NC). Continuous secondary endpoints that were normally distributed are presented as mean  $\pm$  SE and analysed using an unpaired *t*-test. Discrete secondary endpoints were analysed using a chi-square analysis or a Fisher's Exact Test in case of small numbers of events. Lp-PLA<sub>2</sub> was not normally distributed and therefore presented as median with interquartile range and analysed with a Mann-Whitney-U test.

Based on previous studies and observational data from our own clinic, we calculated that a sample size of 80 patients (40 patients per treatment group) would provide a power of 90% with an alpha of 0.05 to detect a 50% reduction in post-operative troponin release. An expected effect size of 50% was based on previous observations on the effect of alternative preconditioning strategies on plasma troponin concentration after CABG<sup>3</sup> or PTCA<sup>29</sup> and on protective effect of preconditioning strategies on infarct size in preclinical models of myocardial infarction.<sup>5</sup> Since this was a *proof of principle* study, a per-protocol analysis was used to assess the primary endpoint.

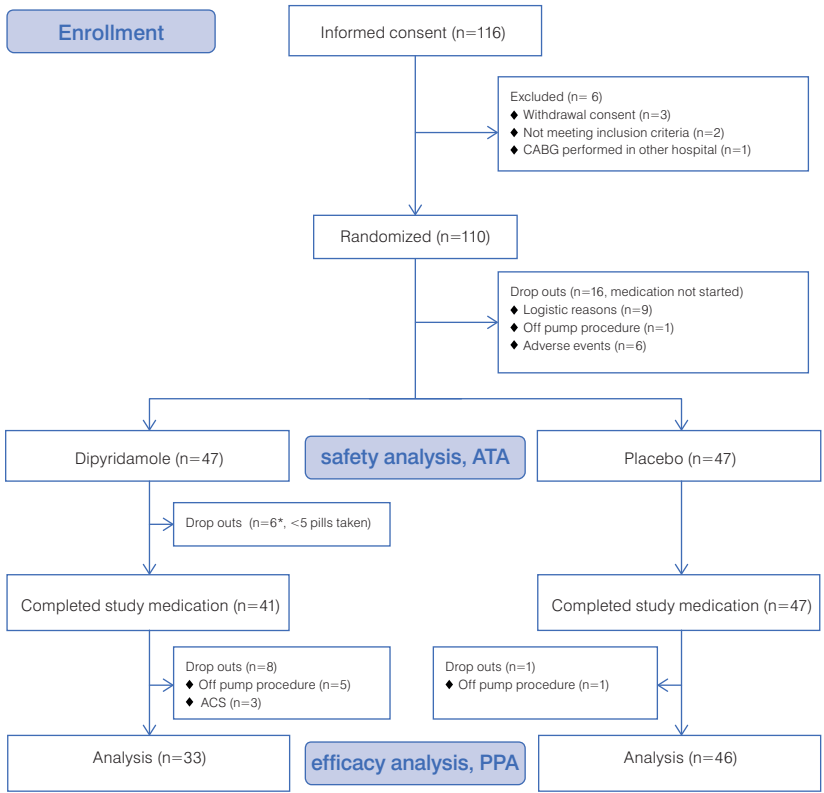
The efficacy analysis was performed both with and without patients with the 34C>T variant of the *AMPD1* gene which has previously been shown to limit the pharmacological action of dipyridamole on adenosine kinetics.<sup>19</sup> Safety analysis was conducted in all patients exposed to study medication (*as treated* analysis), also including

patients that were included in the trial but in whom the surgeon changed plans from 'on-pump' to 'off-pump' while patients were already on investigational product (figure 1).

## Results

### Inclusion

In all, 110 patients provided informed consent and 33 patients (dipyridamole) vs. 46 patients (placebo) were included in the per-protocol analysis (figure 1). Baseline characteristics did not differ between the two treatment arms (table 1). The mean duration of aortic cross-clamping was  $65 \pm 19$  (range: 18-107) minutes, this was not



**Figure 1** Study flow chart.

ATA = as-treated analysis; PPA = per-protocol analysis.

\*:  $p < 0.05$  versus placebo arm (two-sided pearson chi-square analysis)



**Table 1** Baseline characteristics.

	Dipyridamole (N=33)	Placebo (N=46)
Age mean	62.7 (8.3)	63.7 (8.1)
Male	31	37
Body weight, kg	88.4 (9.3)	81.3 (12.7)
Height, cm	177 (7)	174 (10)
Blood pressure, mmHg		
Systolic	138 (18)	141 (15)
Diastolic	81 (10)	80 (9)
Heart rate, beats/min	60 (10)	58 (8)
Previous myocardial infarction	12 (36.4%)	14 (30.4%)
Previous CABG	1 (3.0%)	0 (0%)
Previous PCI	6 (18.2%)	4 (8.7%)
Angina pectoris CSS class		
I	5 (17%)	7 (15%)
II	13 (39%)	22 (48%)
III	10 (30%)	15 (33%)
IV	5 (15%)	2 (4%)
<b>Co-medication</b>		
Aspirin	32 (97.0%)	45 (97.8%)
β-blocker	29 (87.9%)	46 (100%)
ACE- inhibitor	12 (36.4%)	20 (43.5%)
AT2 receptor antagonist	5 (15.2%)	3 (6.5%)
Diuretic	8 (24.2%)	4 (8.7%)
Calcium channel blocker	12 (36.4%)	9 (19.6%)
Nitrates	18 (54.5%)	26 (56.5%)
Statin	29 (87.9%)	41 (89.1%)
<b>Laboratory values at screening</b>		
Total cholesterol, mmol/l	4.57 (0.92)	4.63 (0.92)
Triglycerides (non-fasting), mmol/l	2.37 (1.45)	1.96 (0.83)
HDL cholesterol, mmol/l	1.04 (0.23)	1.08 (0.23)
LDL cholesterol, mmol/l	2.48 (0.71)	2.67 (0.82)
Creatinin μmol/l, median (IQR)	84.0 (75.0 - 93.5)	76.0 (69.8 - 87.3)
GFR (MDRD) ml/1.73 m <sup>2</sup> , median (IQR)	81.0 (69.5 - 89.0)	82.0 (74.0 - 89.0)
Glucose, mmol/l	6.48 (1.65)	6.32 (1.85)
<b>AMPD genotype</b>		
CC	27	37
CT	5	9
TT	1	0
Number of investigational pills taken, median (IQR)	7.0 (6.0 - 9.0)	6.0 (6.0 - 8.3)

Data are number (%) or mean (SD) unless stated otherwise. CSS= Canadian Cardiovascular Association. ACE= angiotensin converting enzyme. AT2= Angiotensine II.

**Table 2** Details of CABG procedure.

	Dipyridamole (N=33)	Placebo (N=46)
Type of anesthesia		
Midazolam/rocuronium/sufentanyl/propofol	20 (60.6%)	14 (30.4%)
Midazolam/rocuronium/sufentanyl	13 (39.4%)	32 (69.6%)
Sevoflurane	20 (60.6%)	36 (78.3%)
Sufentanyl dose during surgery (µg), mean (SD)	441 (112)	399 (131)
Cross clamping time, minutes, mean (range)	64 (29-99)	66 (18-107)
Cardioplegic solution (%)		
Crystalloid	26 (78.8%)	35 (76.1%)
Blood	7 (21.2%)	11 (23.9%)
Logistic Euroscore, median (IQR)	1.4 (1.1 - 2.3)	1.6 (1.1 - 2.2)
Number of grafts, median (IQR)	2.0 (2.0 - 2.0)	2.0 (2.0 - 2.0)
Number of distal anastomoses, median (IQR)	4.0 (3.0 - 4.5)	4.0 (3.0 - 5.0)

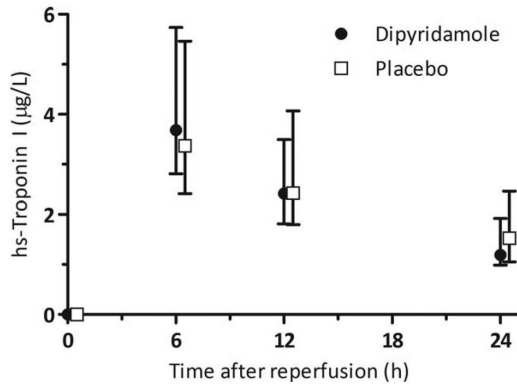
different between groups (dipyridamole  $64 \pm 18$  (29- 99) minutes and placebo group  $66 \pm 19$  (18- 107) minutes,  $p > 0.1$ ). All patients received at least 1 arterial graft (internal mammary artery). Further surgical details are described in table 2.

### Plasma concentration of dipyridamole and caffeine

Immediately before surgery, the plasma dipyridamole concentration averaged  $1.29 \pm 0.11 \mu\text{g/ml}$  for the dipyridamole pre-treated group (range: 0.11 - 2.28  $\mu\text{g/ml}$ ) and  $0.0 \pm 0.0 \mu\text{g/ml}$  for the placebo group. The plasma caffeine concentration was 0.0  $\mu\text{g/ml}$  in all patients, except for two in whom a low concentration was detected (0.17 and 0.22  $\mu\text{g/ml}$ ). These patients were not excluded from analysis since these low concentrations do not relevantly antagonize adenosine receptors.

### Plasma troponin I

Baseline concentrations of hs-troponin I prior to surgery were below the detection limit of 0.02  $\mu\text{g/L}$  in the majority of patients. In 11 patients baseline hs-troponin levels were  $> 0.02 \mu\text{g/L}$  (four patients in the dipyridamole group and seven in the placebo group;  $p > 0.1$ ), with a range of the hs-troponin of 0.02-0.11  $\mu\text{g/L}$  and 0.02-0.24  $\mu\text{g/L}$ , respectively. In three patients these data were missing (one dipyridamole and two placebo). Peak high-sensitive troponin I was 3.7 (2.8-5.7)  $\mu\text{g/L}$  in the dipyridamole group and 3.4 (2.4-5.5)  $\mu\text{g/L}$  in the placebo group at 6 hours after release of the aortic clamp (figure 2). Dipyridamole did not significantly affect post-operative hs-troponin I concentration (change in plasma hs-troponin I -3%, 95% confidence interval [CI] from -23% to 36%;  $p > 0.1$ ). The *AMPD1* 34C>T CT and TT genotype (rs17602729), associated with reduced AMPD activity, was found in six patients in the dipyridamole



**Figure 2** Post-operative hs-troponin I concentration in plasma (median with interquartile range).

Solid line: dipyridamole; Dashed line: placebo. The two curves do not differ significantly (Mixed model analysis after log transformation,  $p > 0.1$ ).

group and in nine patients in the placebo group. When analysis of the course in plasma hs-troponin concentration was restricted to those individuals with the CC genotype, results remained essentially unchanged (data not shown).

Duration of ischemia, defined as aortic cross-clamp time ( $65 \pm 19$ ; range 18-107 minutes), was positively correlated with the amount of troponin release ( $r=0.3$ ,  $p < 0.01$ , Spearman correlation coefficient), supporting the assumption that the postoperative rise in hs-troponin is, at least partly, due to IR-injury.

In the dipyridamole group, the plasma dipyridamole concentration did not correlate with post-operative hs-troponin levels (Spearman correlation coefficient -0.15 and -0.13 for average and maximum hs-troponin concentration, respectively,  $p > 0.1$ ).

### Markers of inflammation

Prior to treatment, the dipyridamole and placebo group did not significantly differ in plasma Lp-PLA<sub>2</sub> concentration (0.57 [0.32-3.09] and 0.74 [0.30 – 1.57] µg/ml, respectively;  $p > 0.1$ , Mann-Whitney *U*-test). In the dipyridamole-treated patients, the plasma Lp-PLA<sub>2</sub> concentration was significantly reduced during drug treatment from baseline by 0.23 (0.05 - 0.83) µg/ml versus 0.03 (-0.13- 0.28) µg/ml in the placebo-treated individuals ( $p=0.007$  for comparison of the change from baseline between the two treatment arms, Mann-Whitney-*U* test). Other inflammatory markers that were

Table 3 Inflammatory markers.

	Dipyridamole		Placebo		p- value*
	Baseline	Effect of treatment	Baseline	Effect of treatment	
LpPLA <sub>2</sub> , µg/ml	0.57 (0.32-3.09)	-0.23 (-0.83 to -0.05)	0.74 (0.30- 1.57)	-0.03 (-0.28 to 0.13)	0.007
IL-6, pg/ml	3.0 (3.0-3.0)	0.00 (0.00 to 0.00)	3.0 (3.0- 3.0)	0.0 (0.0 to 0.0)	0.77
Hs-CRP, mg/L	1.59 (0.83-5.10)	-0.30 (-0.66 to 0.44)	1.14 (0.52- 2.57)	-0.42 (-0.99 to 0.00)	0.37
MMP-9, ng/ml	622.0 (348.50- 865.0)	-228.0 (-311.5 to 71.5)	486.50 (337.5- 703)	-56.50 (-205.75 to 78.25)	0.13
MCP-1, pg/ml	615.0 (430.50-801.50)	47.0 (-54.0 to 126.0)	587.50 (445- 732)	16.50 (-53.75 to 79.25)	0.32
PTX-3, ng/ml	0.52 (0.05- 0.86)	0.00 (-0.09 to 0.02)	0.50 (0.05- 0.73)	0.0 (-0.02 to 0.11)	0.26
Adiponectin, ng/ml	1649.0 (1360.50-2481.0)	-122.0 (-297.50 to -3.50)	1721 (1194.75- 2221.25)	-135.50 (-505.50 to 68.75)	0.51

Data shown as median (IQR). Lp-PLA<sub>2</sub>= Lipoprotein associated phospholipase A2, IL-6= interleukin 6, Hs-CRP= high-sensitive CRP, MMP-9= Matrix metalloproteinase 9, MCP-1= Monocyte chemoattractant protein-1, PTX-3= Pentraxin-related protein 3. \* P-value is calculated by performing a Mann-Whitney U test comparing the change from baseline (effect of treatment) for both treatment groups.

explored (IL-6, hs-CRP, MMP-9, MCP-1, PTX-3, adiponectin) did not change significantly in response to dipyridamole (table 3).

**Table 4** Secondary endpoints.

	Dipyridamole (N=33)	Placebo (N=46)	p-value
Days on ICU, median (IQ range)	1 (1-1)	1 (1-1)	0.71
Number of patients on inotropy	17 (52%)	21 (46%)	0.52
Duration of inotropic support , hours, median (IQ range)	3.5 (0-14.3)	(0-15)	0.79
Clinical relevant arrhythmias < 24 hours	24 (72.7%)	35 (76.1)	0.74
None	7 (21.2%)	7 (15.2%)	0.49
AF/SVT de novo	1 (3%)	3 (6.5%)	0.64
Sinus bradycardia in need for pacemaker	0 (0%)	1 (2.2%)	0.58
AV conduction disturbance	1 (3%)	0 (0%)	0.42
VT/VF			

ICU= intensive care unit. AF= atrial fibrillation. SVT= supraventricular tachycardia. AV= atrioventricular. VT= ventricular tachycardia. VF= ventricular fibrillation.

### Arrhythmias, ICU-stay and need for inotropic support

Dipyridamole did not affect the occurrence of arrhythmias, the need for inotropic support or the duration of ICU stay (table 4).

### Safety

There was no mortality within the first 30 days after surgery. In the dipyridamole group an acute coronary syndrome (ACS) occurred in 3 patients versus none in the placebo group ( $p > 0.05$ , Fisher's Exact Test). In two of these cases, ACS resulted from a complicated surgical procedure: stenosis of an anastomosis and an endarterectomy of the left anterior descending artery. Since these patients did not experience appropriate reperfusion after release of the aortic clamp, they were not included in the per-protocol analysis. The third case of ACS occurred in the morning prior to surgery. Since this complication confounded plasma hs-troponin, this patient was also excluded from the per-protocol analysis.

Clinically relevant bleeding complications occurred in four patients, all on dipyridamole ( $p > 0.10$ , Fisher's Exact Test). In three a causal role for dipyridamole could not be excluded, whereas a fourth bleeding complication was related to a clear surgical complication. Total chest tube output was available in 38 patients on dipyridamole

and 41 patients on placebo and did not significantly differ between the two study arms 785 (620 – 1340) mL and 880 (558-1215) mL in dipyridamole and placebo group; respectively; median (interquartile range);  $p > 0.1$ , Mann-Whitney-U test). One patient (on dipyridamole) experienced a delirium post-surgery which prolonged hospital stay. In total, 31 patients experienced a headache (22 on dipyridamole, 9 on placebo;  $p < 0.005$ , Pearson chi-square test). In most cases the headache occurred immediately after intake of study medication. Four patients (two on dipyridamole, two on placebo,  $p = 1.0$ ) experienced chest pain without increase in plasma cardiac enzymes. Finally, 19 patients experienced various adverse events without further clinical significance (13 in the dipyridamole group and 6 in the placebo group and,  $p < 0.10$ , Pearson chi-square Test). From 47 patients who started dipyridamole treatment, in 43 patients the plasma dipyridamole concentration was available (in 3 who took  $< 5$  pills and 1 who was shifted to off-pump surgery, dipyridamole concentration was missing). The dipyridamole concentration did not significantly differ between those who developed any adverse event and those who did not ( $1.3 \pm 0.2 \mu\text{g/ml}$  ( $n = 27$ ) and  $1.2 \pm 0.2 \mu\text{g/ml}$  ( $n = 16$ ) respectively;  $p > 0.5$ ,  $t$ -test). Similar results were obtained when this analysis was restricted to those who reported headache.

## Discussion

In this study we investigated whether short-term treatment with dipyridamole could reduce IR-injury in patients undergoing elective CABG surgery. Although preclinical data suggested a cardioprotective effect of dipyridamole, in our study dipyridamole did not limit myocardial IR-injury, as detected with post-operative plasma hs-troponin I release. Also, there was no effect on the occurrence of post-operative arrhythmias, the need for inotropic support or the duration of ICU stay. This lack of efficacy was combined with the occurrence of adverse events: mainly headaches and possibly also an increase in bleeding risk, although overall blood loss was not significantly affected.

Dipyridamole is a potent inhibitor of the human ENT transporter (hENT1).<sup>30</sup> Inhibition of the hENT1 increases the interstitial concentration of adenosine and subsequent adenosine receptor stimulation<sup>16,31</sup> which is an attractive target to prevent IR-injury.<sup>32</sup> Dipyridamole reduced myocardial and cerebral infarct size in animals<sup>12-15</sup> and reduced IR-injury in humans in-vivo.<sup>11, 16-18</sup> In contrast to our hypothesis, we did not observe any benefit of dipyridamole on plasma hs-troponin I after CABG. We will subsequently discuss methodological, pharmacokinetic and pharmacodynamic explanations for this unexpected result.

The drop-out rate was higher during dipyridamole treatment (13 patients) than during placebo treatment (one patient). This was mainly driven by differences in adherence to therapy between the two groups, most likely resulting from side effects to dipyridamole including headache. In theory, this drop-out could have occurred selectively in those individuals that would otherwise have responded well to dipyridamole therapy, for example by increased plasma exposure due to higher bioavailability of dipyridamole in this subset of patients. However, adverse events also occurred in those individuals who completed therapy. In these, the presence of adverse events was not associated with higher plasma dipyridamole concentration (data not shown). Furthermore, plasma hs-troponin (primary endpoint) did not differ between those who experienced adverse events and those who did not. Therefore, potential bias due to selective drop-outs in the dipyridamole group does not sufficiently explain the negative result of this trial.

Oral therapy with dipyridamole could have resulted in insufficient plasma dipyridamole concentrations. In a previous study in healthy volunteers by our group, oral dipyridamole achieved an average plasma peak concentration of 1.33 (SD 0.33)  $\mu\text{g/mL}$ , significantly inhibited *ex-vivo* transmembranous adenosine transport in erythrocytes and reduced forearm IR-injury as imaged with annexin A5 scintigraphy.<sup>11</sup> In our patients, average dipyridamole concentrations were 1.34  $\mu\text{g/mL}$ , but ranged from 0.11 - 3.04  $\mu\text{g/mL}$ . Thus, the group average concentration well resembled the previously reported value in healthy volunteers, but dipyridamole exposure was variable in our patients and may have been insufficient in some of them to prevent IR- injury. Dipyridamole concentrations, however, did not directly correlate with post-operative troponin levels.

We have previously shown that a genetic polymorphism resulting in reduced AMPD1 activity (CT and TT genotype) limits the efficacy of dipyridamole to augment adenosine signaling during ischemia.<sup>19</sup> Exclusion of patients with CT or TT genotype, however, did not alter the results of our trial. Therefore, this genetic variant did not mask a potential benefit of dipyridamole on our primary endpoint.

Our patient population is characterized by high age, co-morbidity (such as hypertension) and the use of comedication such as statins. This obviously differs from the healthy volunteers that we have studied previously as well as from animal models that showed benefit from dipyridamole. We have recently shown that the efficacy of ischemic preconditioning to prevent brachial endothelial dysfunction after ischemia and reperfusion is reduced in the elderly.<sup>33</sup> Since adenosine is involved in ischemic preconditioning, the reduced efficacy of this intervention in the elderly may reflect a reduced efficacy of endogenous adenosine to protect tissue against IR-injury and

may therefore also limit the efficacy of dipyridamole to reduce IR-injury. Furthermore, we and others have shown that statins augment adenosine signaling by activating ecto-5'-nucleotidase and prevent IR-injury in animals as well as healthy human volunteers and patients.<sup>34-36</sup> Therefore, the almost universally used statins could have maximized the potential efficacy of endogenous adenosine to prevent IR-injury and this could have prevented dipyridamole from providing additional benefit. In addition, general translational failure of promising preclinical findings might be explained by methodological flaws in animal studies, e.g., lack of randomization, lack of blinding, lack of planning for missing data, and a willingness to accept post-hoc data analyses with results in hand.<sup>37</sup>

Finally, additional actions of dipyridamole could have offset the benefit of augmenting adenosine signaling. Interestingly, our exploratory data on Lp-PLA<sub>2</sub> show that dipyridamole significantly reduces the plasma concentration of this enzyme. Lp-PLA<sub>2</sub> generates pro-inflammatory lipid molecules (such as lyso-phosphatidylcholine) from oxidized LDL particles.<sup>38, 39</sup> Epidemiological data have shown a strong association between elevated levels of Lp-PLA<sub>2</sub> and the risk of acute cardiovascular events in humans.<sup>40-42</sup> In animal models, pharmacological inhibition of Lp-PLA<sub>2</sub> reduces atherosclerotic plaque formation.<sup>43</sup> Lp-PLA<sub>2</sub>-knock-out animals show less formation of atherosclerosis.<sup>44</sup> Finally, in humans with coronary atherosclerotic disease, pharmacological inhibition of Lp-PLA<sub>2</sub> prevents development of signs of plaque instability on intravascular ultrasound.<sup>45</sup> Thus, reduction of plasma Lp-PLA<sub>2</sub> concentration and subsequent prevention of generation of vulnerable plaques could contribute to the previously observed benefit of dipyridamole to prevent atherothrombotic events.<sup>46</sup> However, Lp-PLA<sub>2</sub> is also involved in the degradation of Platelet Activating Factor, a substance that augments IR-injury in various animal models. This action of dipyridamole could therefore have offset the potential benefit of augmented adenosine signaling to prevent cardiac IR-injury.<sup>47-50</sup>

In conclusion, in contrast to our hypothesis and previous preclinical studies, we could not detect any benefit from oral dipyridamole treatment on IR-related myocardial injury during CABG. At least three possible explanation may contribute to this negative finding: variable plasma exposure to dipyridamole, reduced benefit of augmented adenosine signaling in this population of elderly patients with co-morbidity and comedication and additional actions of dipyridamole including reduction of plasma Lp-PLA<sub>2</sub> concentration.



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# 7

## Ticagrelor does not inhibit adenosine transport at relevant concentrations: A randomized cross-over study in healthy subjects in vivo

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## Introduction

Ticagrelor is a novel direct-acting and reversibly binding P2Y<sub>12</sub> receptor antagonist. In the Platelet Inhibition and Patient Outcomes (PLATO) trial, the administration of ticagrelor to patients with an acute coronary syndrome resulted in a striking reduction in the primary endpoint of death from vascular causes, myocardial infarction, or stroke compared to clopidogrel.<sup>1</sup> Moreover, all cause mortality was reduced, and this was driven not only by vascular mortality, but also by fewer deaths attributed to sepsis.<sup>2</sup> In addition, dyspnea and asymptomatic ventricular pauses were reported more often in the ticagrelor treated patients. These findings have instigated the search for pleiotropic effects of ticagrelor over and above the classical antiplatelet effect.

In the past years, evidence is accumulating that ticagrelor inhibits the cellular uptake of the endogenous nucleoside adenosine by blockade of the Equilibrative Nucleoside Transporter (hENT1).<sup>3</sup> Stimulation of membrane-bound adenosine receptors induces various beneficial cardiovascular effects, including vasodilation, inhibition of platelet aggregation, inhibition of inflammation, and increasing resistance against ischemia and reperfusion.<sup>4, 5</sup> In addition, intravenous administration of adenosine induces dyspnea and potently inhibits atrioventricular conductance.<sup>5, 6</sup> Adenosine is mainly formed by intra- and extracellular dephosphorylation of AMP, which is catalyzed by 5'-nucleotidase. In contrast, degradation of adenosine in humans is mainly confined to the intracellular compartment, by adenosine kinase and adenosine deaminase. Consequently, in normal situations, the intracellular concentration is lower than the extracellular concentration, and extracellular adenosine is rapidly taken up via the hENT1 by surrounding cells, mainly red blood cells and endothelial cells.<sup>7</sup> Therefore, inhibition of the hENT1 activity will increase the extracellular adenosine concentration, and this mechanism has been proposed to mediate the effects of ticagrelor observed in the PLATO study.<sup>3</sup>

Most of the current evidence on the effect of ticagrelor on adenosine metabolism is derived from *in vitro* studies, or from studies in patients in which the adenosine metabolism could be affected by other factors, such as co-medications. Therefore, in this study we aimed to test this hypothesis in healthy humans *in vivo*. We used the vasodilator response to the local administration of adenosine and to forearm ischemia as validated surrogates of adenosine uptake inhibition, as previously described.<sup>8-11</sup> Furthermore, the vasodilator response to dipyridamole, a potent inhibitor of hENT1, was used as a validated surrogate marker of endogenous adenosine formation.<sup>12, 13</sup> In addition, the effect of ticagrelor on adenosine and uridine uptake was investigated directly in isolated red blood cells and whole blood.



## Methods

### Subjects

The study protocol was in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the Radboud University Medical Center, Nijmegen, The Netherlands. After approval, 15 healthy male volunteers signed written informed consent statements before participation in the study, see also figure 1. They had no history of cardiovascular disease, bleeding tendency or asthma, and they were all non-smokers. Concomitant medication was not permitted. In all participants a physical examination, electrocardiography, and laboratory investigation were performed to exclude cardiovascular and pulmonary disease, hypertension, diabetes mellitus, renal dysfunction, liver enzyme abnormalities, and thrombocytopenia.

### Experimental protocol

The subjects were randomly assigned in a double-blinded cross-over design to a single dose of ticagrelor 180 mg (Brilique, 2 tabs of 90 mg, AstraZeneca) or 2 fully mimicking placebos (Apotheek Haagse Ziekenhuizen, the Hague, the Netherlands). The experiments were performed two hours after the intake of the study medication. The two experiments were separated by at least 14 days. The randomization code was kept at the department of Clinical Pharmacy of the Radboud University Medical Centre. The study medication was taken under supervision at the Clinical Research Centre of our hospital.

This trial was prospectively registered at: [clinicaltrials.gov](https://clinicaltrials.gov) (The effect of ticagrelor on the adenosine system; NCT01996735).

### Venous occlusion plethysmography

On the experimental days, we studied the subjects in supine position after an overnight fast. All volunteers were asked to abstain from alcoholic drinks and caffeine-containing beverages for at least 24 hours before the experiments. We performed the experiment in a temperature-controlled room ( $24 \pm 1$  °C). A 27-gauge needle was inserted into the brachial artery of the non-dominant arm for intra-arterial drug administration. We measured the forearm blood flow (FBF) in both forearms simultaneously with venous occlusion plethysmography, using mercury-in-silastic strain gauges and an occluded hand circulation as described previously.<sup>14</sup> The total intra-arterial infused volume was kept at a constant rate of 50  $\mu\text{L}/\text{min}$  per dL of forearm volume. Thirty minutes after cannulation of the brachial artery, we started the infusion of normal saline at the calculated rate with concomitant measurement of the FBF. Normal saline (baseline) and the dosages of the intra-arterial administered drug were infused for 5 minutes. We performed four experiments, starting two hours after intake

of the study medication, which were all separated by a wash-out period of 30 minutes to prevent any cross-over effects. Figure 2 provides a schematic overview of the experiment.

1. We measured FBF during the administration of 4 incremental dosages of adenosine into the brachial artery. The dosages (0.15, 0.5, 1.5, and 5.0  $\mu\text{g}/\text{min}/\text{dL}$  forearm volume) are similar to a previous study in which we demonstrated a significant potentiation of adenosine-induced vasodilation by the ENT inhibitor dipyridamole and drafazine.<sup>9, 10</sup> Adenosine-induced forearm vasodilation is the primary endpoint of our study. Secondary endpoints include:
2. The blood flow response to 2 and 5 minutes of forearm ischemia was measured (post-occlusive reactive hyperemia; PORH). Forearm ischemia was induced by inflation of an upper arm cuff to 200 mmHg, as described previously.<sup>14</sup> We have previously demonstrated that dipyridamole potentiates the PORH response<sup>8</sup> and that statins potentiate the PORH by an increase of the extracellular adenosine formation.<sup>15</sup>
3. Subsequently, FBF was measured during intra-arterial administration of dipyridamole (30 and 100  $\mu\text{l}/\text{min}/\text{dL}$  forearm volume, which we also used in previous studies<sup>11</sup>). Dipyridamole induces vasodilation by ENT inhibition, thereby increasing the extracellular adenosine concentration at a rate proportionate to extracellular adenosine formation.
4. Finally, we measured the FBF response to the administration of acetylcholine (0.5 and 2.0  $\mu\text{g}/\text{min}/\text{dL}$  forearm volume) to exclude nonspecific effects on vascular reactivity.

### Blood sampling

Before intake of the study medication blood was drawn from an intravenous cannula in the dominant arm for the determination of the plasma ticagrelor and caffeine concentration, and for the *ex vivo* ENT transport measurements in isolated red blood cells. This measurement was repeated approximately 2 hours after the ingestion of the study medication and just before measurement of the vasodilator response to adenosine. Finally, the ticagrelor concentration was determined before intake of the ticagrelor dose, 2 hours after the intake, and subsequently before the start of each forearm blood flow experiment.

### Analytic procedures

We determined plasma caffeine concentrations using reversed-phase HPLC with ultraviolet detection set at 273 nm, according to Schreiber-Deturmeny and Bruguerolle.<sup>16</sup> In isolated red blood cells, the uptake of adenosine and uridine was determined as described previously.<sup>17</sup> In contrast to adenosine, uridine is not metabolized inside the cells, and therefore, uridine uptake is a more direct measure of ENT activity than

adenosine uptake.<sup>18</sup> Ticagrelor concentrations were determined by LC-MSMS. Liquid chromatographic separation was performed at a temperature of 30°C with a mobile phase, consisting of solvent A (10 mM ammonium acetate in water) and solvent B (acetonitrile). For the mass spectrometric analysis, heated electrospray ionization (HESI) was operated at a spray voltage of -2.5kV, a capillary temperature of 225°C and a vaporizer temperature of 382°C. Argon was used as collision gas at a pressure of 1.5 mTorr. Negative ion mode was used with selected reaction monitoring (SRM) for the quantitative analysis of ticagrelor. The most abundant product ion was used for quantification. The quantification was performed using peak areas.

### In vitro experiments

In isolated red blood cells from healthy volunteers not taking any medication, the effect of ticagrelor on uridine uptake was measured as described previously.<sup>17</sup> Briefly, uridine was added to washed red blood cells to obtain a final concentration of 1000  $\mu$ M. The cells were pre-incubated with increasing concentrations of ticagrelor for ten minutes before the addition of uridine. After 3 seconds, uridine uptake was completely blocked by the addition of 10  $\mu$ M dipyridamole and the amount of uridine in the cell was determined using HPLC with UV detection set at 254 nm.

Because of the high protein binding of ticagrelor of 99.8%, we also performed whole blood experiments, in which ticagrelor was added to 1 ml of whole blood for 10 or 60 minutes. Next, the red blood cells were isolated by centrifugation and uridine was added as described above.

To measure the rate of disappearance of adenosine in the physiological situation, which is the overall result of uptake and intracellular degradation, we added 1  $\mu$ M of adenosine to 1 ml of whole blood at 37°C, as previously described by Bonello et al.<sup>19</sup> After 0, 15, 30, 45, and 60 seconds, the transport, formation, and degradation of adenosine was completely blocked by adding an equal volume of pharmacological blocking solution, including NaCl (118mM); KCl (5 mM); Na<sub>2</sub>EDTA (13.2 mM); dipyridamole (40  $\mu$ M); iodotubercidin (ITU; 10 $\mu$ M); EHNA (10 $\mu$ M); forskolin (11.5 $\mu$ M), and IBMX (115 $\mu$ M). After centrifugation the adenosine concentration was measured in the supernatant using LC-MSMS. Separation was performed with a Acquity UPLC HSS column. The mobile phase, consisting of solvent A (1 mM ammonium fluoride in water) and solvent B (methanol). For the mass spectrometric analysis, heated electrospray ionization (HESI) was operated at a spray voltage of +3kV, the capillary temperature and the vaporizer temperature were set at 300°C. Argon was used as collision gas at a pressure of 1.5 mTorr. Positive ion mode was used with selected reaction monitoring (SRM) for the quantitation. The following SRM transitions were used:  $m/z$  268.1 (parent ion) to  $m/z$  119.0 and 136.1 (both product ions).

## Statistical analysis

The study was powered to detect a difference in the primary endpoint of adenosine-induced vasodilation. The sample size calculation was based on the following assumptions: in previous studies from our own group, we found that the vasodilator response to the intrabrachial administration of adenosine averages  $2.8 \pm 0.6$ ,  $4.4 \pm 1.0$ ,  $9.0 \pm 1.7$  ml/min per dl of forearm volume for 0.5, 1.5, and 5.0  $\mu\text{g}/\text{min}/\text{dl}$ , respectively (mean  $\pm$  SEM,  $n=8$ ). The pooled CV is 0.6, so after log transformation the SD averaged 0.55. We expect that a (relative) difference between the treatments is independent of the adenosine dose. Hence, a linear mixed model will be used, with fixed factors treatment (ticagrelor vs placebo), adenosine dose, and period. Based on a correlation of 0.7 between the measurements for all doses and time points and an SD of 0.55, the SD of the contrast is 0.25. As a result, 13 evaluable subjects are needed to demonstrate an augmentation of adenosine-induced vasodilation with 1.25 (ie a 25% increase) with a power of 80% and a two-sided alpha of 0.05. To account for one drop-out, we aim to include 14 healthy volunteers.

FBF analyses were done offline before unblinding of the study. Mean FBF values were calculated from the FBF responses to the different stimuli. We calculated the average FBF during last 4 minutes of the baseline FBF (normal saline), the last 2 minutes of the FBF response to adenosine, dipyridamole and acetylcholine, and the first 3 minutes of the PORH. Results are expressed as the median absolute FBF (mL/dL/min) with interquartile range (25-75%), unless otherwise stated.

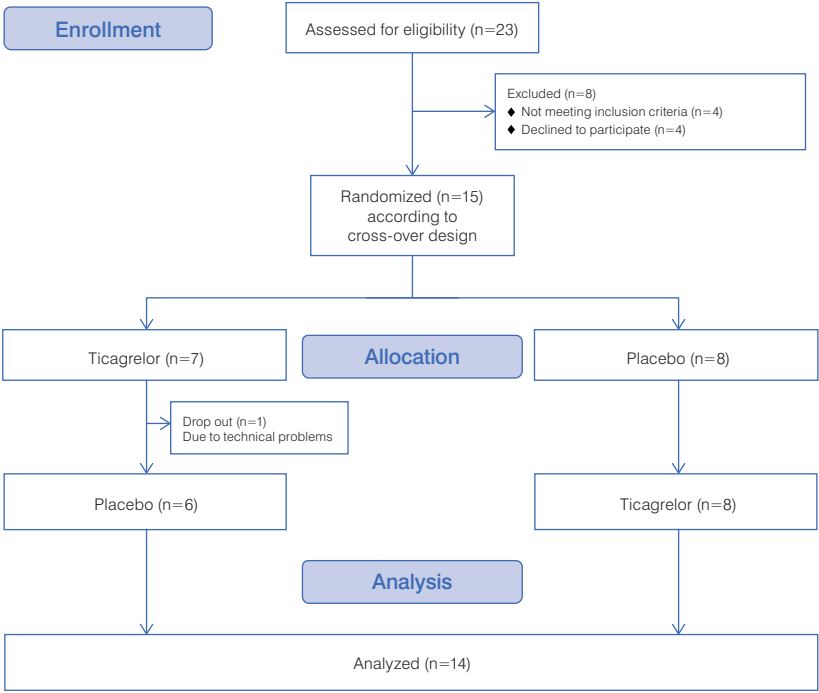
A linear mixed model was used to compare the FBF results of both experiments, with the log FBF during placebo and ticagrelor treatment as the dependent variable, and treatment (ticagrelor versus placebo), adenosine dose, and period as fixed factors. We used a heterogeneous compound symmetry as the type of repeated covariance. A carry over effect was excluded calculating the interaction between 'sequence' and the primary endpoint in the mixed model analysis. For uridine uptake experiments, sigmoidal dose response curves were constructed with a variable slope and  $\text{IC}_{50}$  were calculated for each series of experiments, using GraphPad Prism.

## Results

### Subjects

23 subjects were screened for eligibility. Four participants withdrew from participation and 4 participants were excluded, because of drug abuse, a history of asthma, a platelet count of  $145 \times 10^9/\text{L}$ , and a systolic heart murmur. Insertion of the arterial needle failed in 1 subject and this subject was replaced. Due to repeated dislocation

of the intra-arterial needle, we had to discontinue the experiment after the infusion of dipyridamole during a single visit in another subject. Thus, 13 fully evaluable subjects were included and one subject in whom acetylcholine responses were lacking during one (placebo) visit, see also figure 1.



**Figure 1** Schematic flow chart of the experiment.

The baseline characteristics are depicted in table 1. The plasma caffeine concentration was  $< 1$  mg/L in all subjects, showing adequate caffeine restriction. The ticagrelor concentration averaged  $1.18 \pm 0.13$ ,  $1.01 \pm 0.11$ ,  $0.90 \pm 0.09$ , and  $0.80 \pm 0.08$   $\mu\text{M}$  (mean  $\pm$  SE) at 2, 3, 4, and 4.5 hours after ticagrelor intake, respectively (see figure 2 for the time points).

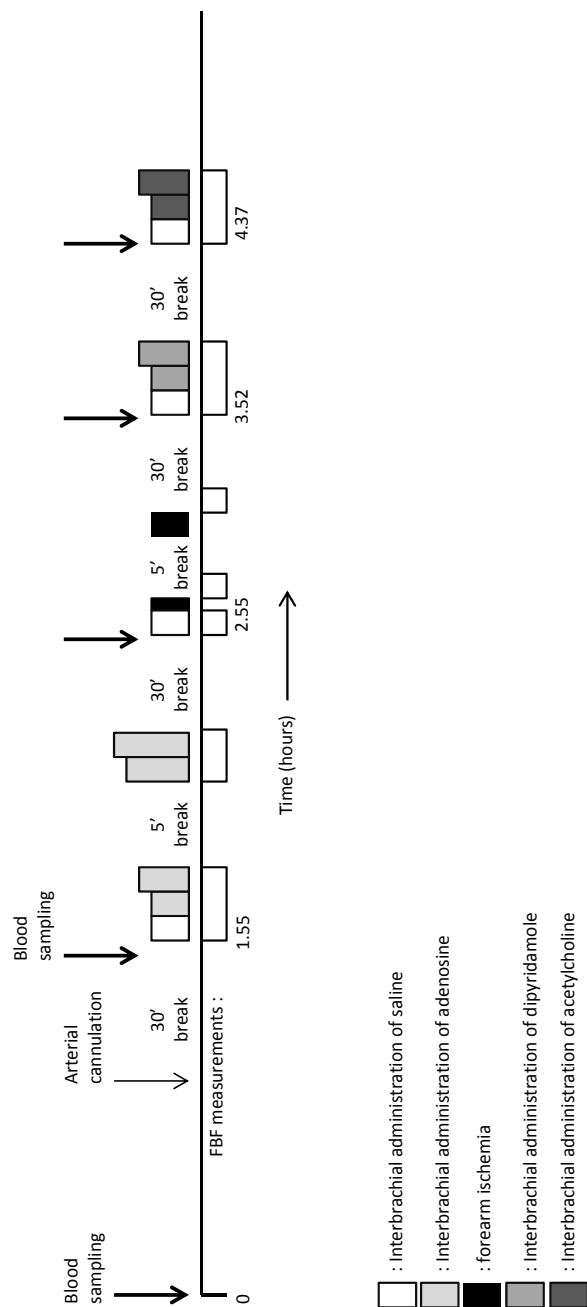
**Table 1** Baseline characteristics (means  $\pm$  SD; n=14).

Characteristic	Value
Age (years)	22.1 $\pm$ 2.2
Blood pressure (mmHg)	
Systolic	125 $\pm$ 7
Diastolic	67 $\pm$ 10
Heart rate (bpm)	62 $\pm$ 10
BMI (kg/m <sup>2</sup> )	22.3 $\pm$ 1.9
Blood plasma values	
Platelet count (*10 <sup>9</sup> /L)	230 $\pm$ 31
Creatinine ( $\mu$ mol/L)	80 $\pm$ 7
Non-fasting glucose (mmol/L)	5.1 $\pm$ 0.7
ALAT (U/L)	29 $\pm$ 14
Non-fasting cholesterol (mmol/L)	3.9 $\pm$ 0.7

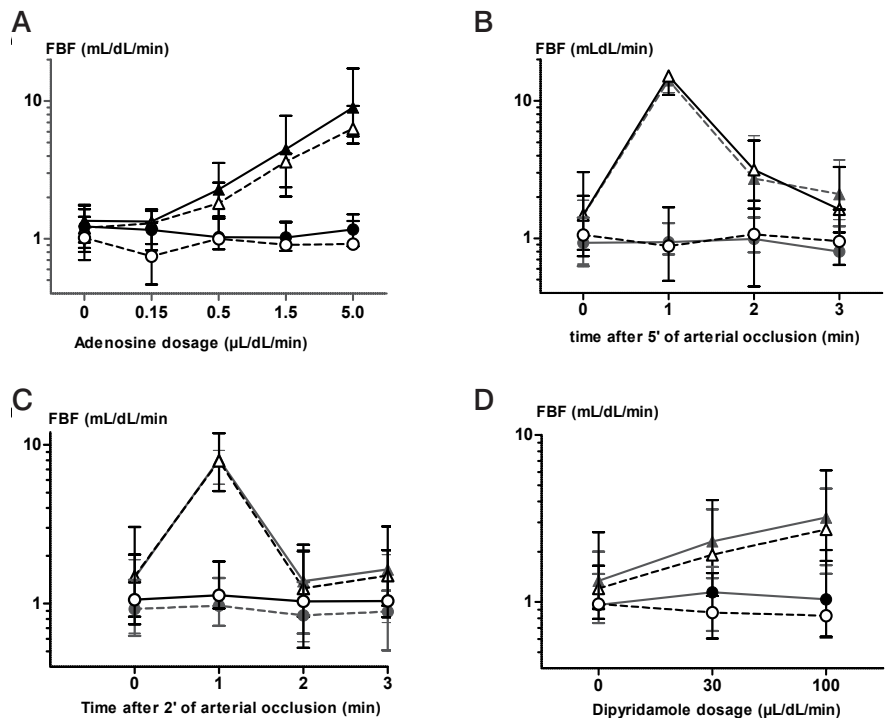
### Venous occlusion plethysmography

Baseline FBF in the experimental forearm was 1.2 (0.9-1.8) and 1.35 (0.9-1.7) mL/dL/min during placebo and ticagrelor, respectively. There was no significant carry-over effect in this study ( $P=0.58$ ). Intrabrachial administration of adenosine significantly increased FBF in the experimental forearm, but not in the control forearm ( $P<0.01$ ; fig. 3A). Pretreatment with ticagrelor did not potentiate adenosine-induced vasodilation: the incremental dosages of adenosine increased the FBF to 1.3 (0.9-1.6), 1.8 (1.4-2.6), 3.6 (2.0-4.1), and 6.3 (4.9-9.2) mL/dL/min during placebo, and to 1.3 (1.2-1.6), 2.3 (1.5-3.6), 4.5 (2.4-7.8), and 9.0 (5.5-17.2) mL/dL/min after ticagrelor administration ( $P=0.33$ ; figure 3A). Furthermore, we did not see any correlation between the plasma ticagrelor concentrations and the area under the curve for adenosine-induced FBF (Spearman correlation coefficient -0.13,  $P=0.67$ ; figure 4).

Baseline FBF before arterial occlusion was 1.5 (0.8-3.0) mL/dL/min during placebo and 1.5 (0.6-1.9) mL/dL/min during ticagrelor treatment. The hyperemic response after 2 minutes of arterial occlusion was 7.9 (5.1-11.8), 1.2 (0.8-2.3), and 1.5 (1.0-3.1) during placebo and 8.1 (5.6-9.2), 1.4 (0.6-2.2), and 1.6 (0.8-2.0) during ticagrelor in the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> minute respectively ( $p=0.96$  for the effect of ticagrelor) (figure 3B).



**Figure 2** Study Flow chart.

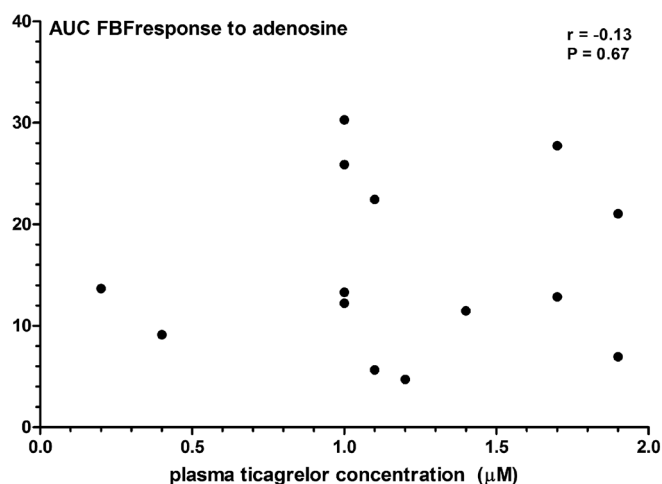


**Figure 3** Forearm blood flow measurements.

FBF values in the experimental forearm (triangle) and non-experimental forearm (circle) after ticagrelor treatment (black symbols) or placebo treatment (open symbols, dashed line) during intrabrachial administration of adenosine (A), after five (B) and two (C) minutes of forearm ischemia, and during intrabrachial administration of dipyridamole (D). P-values represent the effect of ticagrelor on the FBF-values.

After 5 minutes of arterial occlusion, the hyperemic response was 15.2 (11.1-23.7), 3.1 (1.6-5.1), and 1.6 (1.1-3.3) mL/dL/min during placebo and 14.1 (11.5-20.3), 2.7 (1.9-5.6), and 2.1 (1.4-3.7) mL/dL/min in the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> minute respectively ( $p=0.20$  for effect of ticagrelor; figure 3C).





**Figure 4** The correlation between the plasma ticagrelor concentration and the area under the curve (AUC) of adenosine-induced forearm vasodilation.

Comparable to adenosine, intrabrachial administration of dipyridamole significantly increased FBF in the experimental forearm but not in the control forearm ( $P < 0.01$ , figure 3D). Again, the vasodilator response to dipyridamole did not differ between placebo and ticagrelor treatment. The FBF in the experimental forearm was 1.2 mL/dL/min (0.9-2.6) at baseline and 1.9 (1.1-4.1) and 2.7 (1.8-6.1) during administration of the two doses of dipyridamole after treatment with placebo, versus 1.3 (0.9-2.0), 2.3 (1.4-3.6), and 3.2 (1.5-4.8) after treatment with ticagrelor ( $p = 0.29$  for effect of ticagrelor, figure 3D).

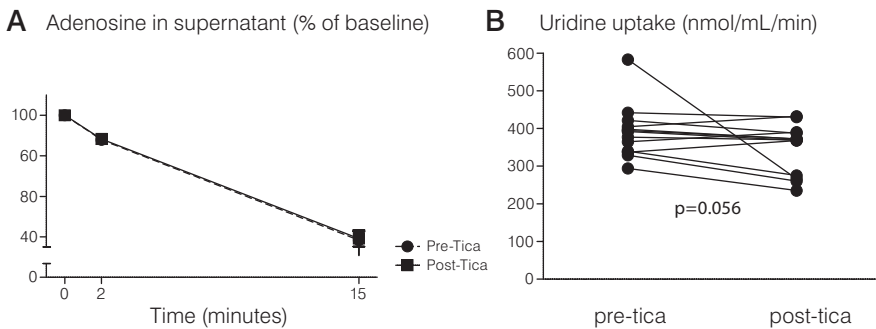
Similarly, ACh-induced vasodilation did not differ between both experiments. The FBF at baseline and during administration of the two doses of acetylcholine was 3.5 mL/dL/min (2.0-5.3), 10.8 (7.8-19.1), and 17.3 (12.5-31.2) after treatment with placebo, versus 3.9 (3.0-6.5), 8.1 (4.4-19.3), and 16.8 (10.4-28.6) after treatment with ticagrelor ( $p = 0.11$  for effect of ticagrelor).

### Ex vivo nucleoside uptake inhibition

Red blood cells were isolated from blood samples taken before and 2 hours after intake of the study drug. The uptake of adenosine in these cells was not affected by oral ticagrelor, when compared to baseline ( $P = 0.87$ ; Fig 5A). Uridine uptake was inhibited by an average of 11%, but this did not reach statistical significance ( $P = 0.056$ ) (Fig 5B).

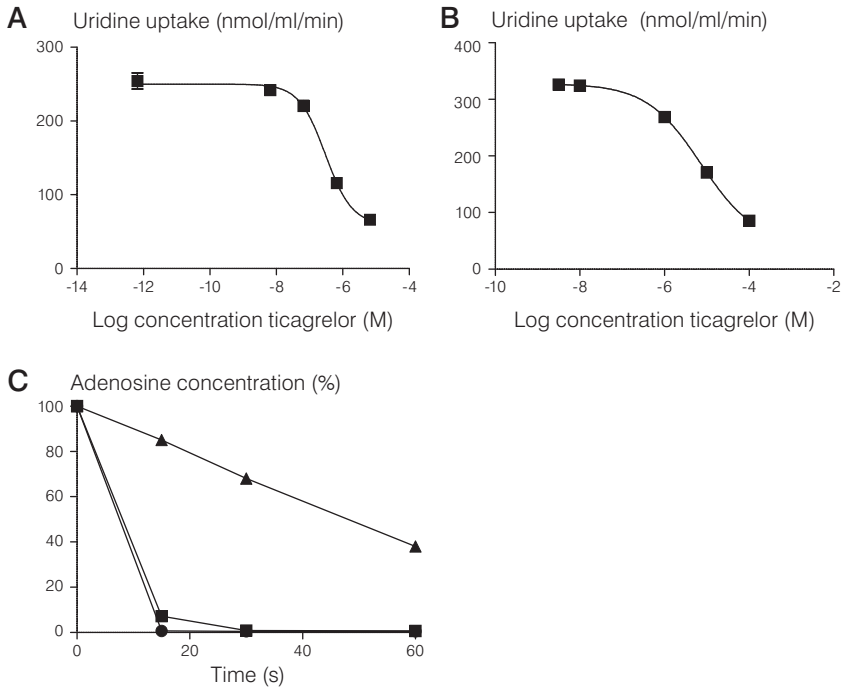
### In vitro nucleoside uptake inhibition

In isolated red blood cells, ticagrelor dose-dependently inhibited uridine uptake with an  $IC_{50}$  value of  $3.0 \times 10^{-7}$  M (95% CI  $2.0 \times 10^{-7}$  to  $4.5 \times 10^{-7}$ ,  $n=3$ ; Fig 6A). In the experiments in which ticagrelor was added to whole blood before isolation of the red blood cells, the  $IC_{50}$  of ticagrelor for uridine uptake inhibition was  $7.3 \times 10^{-6}$  M (95% CI  $6.0 \times 10^{-6}$  to  $1.3 \times 10^{-5}$ ,  $n=3$ ; Fig 6B). In addition, in whole blood, the disappearance of adenosine was only inhibited significantly by the highest concentration of ticagrelor used ( $10^{-4}$  M) ( $n=2$ ; Fig 6C).



**Figure 5** *Ex vivo* uptake of adenosine (**A**) and uridine (**B**) in red blood cells isolated from the subjects before (pre-tica) and two hours after intake of ticagrelor (post-tica).

In A, the adenosine concentration in the supernatant is expressed as the percentage of the baseline adenosine concentration ( $n=13$ ;  $P>0.1$ ). In B, the uridine uptake is expressed for each individual subjects before and after intake of ticagrelor ( $n=13$ ,  $P=0.056$  for comparison between before and after).



**Figure 6** Forearm blood flow measurements.

FBF values in the experimental forearm (triangle) and non-experimental forearm (circle) after ticagrelor treatment (black symbols) or placebo treatment (open symbols, dashed line) during intrabrachial administration of adenosine (A), after five (B) and two (C) minutes of forearm ischemia, and during intrabrachial administration of dipyridamole (D). P-values represent the effect of ticagrelor on the FBF-values.

## Discussion & conclusions

The main finding of our study is that in healthy subjects a single dose of 180 mg of ticagrelor does not potentiate the forearm vasodilator response to adenosine, nor post-occlusive reactive hyperemia and dipyridamole-induced vasodilation. In addition, we observed no significant *ex vivo* nucleoside uptake inhibition in isolated red blood cells in these subjects after oral treatment with ticagrelor. These *in vivo* and *ex vivo* findings are consistent with additional *in vitro* studies in isolated red blood cells demonstrating that ticagrelor only inhibits nucleoside transport in concentrations that

are considerably higher than those obtained after normal dosing. Together, our findings argue against a role for increased adenosine receptor stimulation as a relevant pleiotropic effect of ticagrelor.

In the last few years, several papers suggest that ticagrelor increases the extracellular concentration of endogenous adenosine and it is speculated that this contributes to the effects of ticagrelor that are observed in clinical studies that cannot easily be explained by platelet aggregation inhibition, including reduced mortality, dyspnea, ventricular pauses, limitation of myocardial infarct size, and effects on inflammation.<sup>3, 20</sup> Adenosine induces various effects by stimulation of membrane-bound adenosine receptors, including direct vasodilation, platelet aggregation inhibition, modulation of sympathetic nervous system activity, modulation of inflammation, and limitation of (myocardial) ischemia and reperfusion injury.<sup>4, 5</sup> In addition, the intravenous administration of adenosine has negative dromotropic effects on the heart, and induces a sensation of dyspnea.<sup>5, 6</sup>

Previous *in vitro* studies have demonstrated two different effects of ticagrelor on adenosine metabolism: first, ticagrelor augments the release of adenosine triphosphate (ATP), which acts as a precursor for extracellular adenosine formation, from isolated red blood cells, with an  $IC_{50}$  value of 14  $\mu M$ .<sup>21</sup> Secondly, ticagrelor inhibits adenosine uptake via the ENT with a reported  $IC_{50}$  value of 100-260 nmol/l.<sup>22, 23</sup> It needs to be emphasized, however, that these experiments were performed in isolated cells without plasma. Given the > 99% plasma protein binding of ticagrelor in human blood, these  $IC_{50}$  values cannot easily be compared to the whole blood ticagrelor concentration of 0.5-1.5  $\mu M$  observed during normal dosing in patients. Ticagrelor does not have a direct effect on adenosine receptors itself, does not affect the breakdown of adenosine by adenosine deaminase, nor is it converted into adenosine.<sup>19</sup>

Our experiment was designed to investigate both the effect of ticagrelor on adenosine uptake as well as the effect of ticagrelor on extracellular adenosine formation (e.g. by increased ATP release). Direct measurement of the extracellular adenosine concentration is technically highly demanding given the extremely short half-life of adenosine and there is great controversy about the normal extracellular concentration in human plasma.<sup>24</sup> Therefore, we used well-validated surrogates of extracellular adenosine formation and uptake. In the absence of any effects of ticagrelor on adenosine receptors or adenosine degradation, the vasodilator response to the intrabrachial administration of adenosine is a well-validated surrogate of adenosine transporter function, as we have previously described.<sup>9, 10</sup> We also studied PORH because this is mediated, at least in part, by an increased endogenous formation of adenosine.<sup>8</sup> Dipyridamole is a potent hENT1 blocker and increases the extracellular

adenosine concentration at a rate proportional to the extracellular formation of adenosine, as demonstrated previously.<sup>12</sup> We did not observe an effect of ticagrelor on any of these stimuli, demonstrating that there is no relevant increase in extracellular adenosine formation and adenosine transport. Nonspecific effects of ticagrelor on vascular reactivity were ruled out using acetylcholine-induced vasodilation. Consistent with these *in vivo* findings, there was no significant inhibition of *ex vivo* adenosine uptake in red blood cells, isolated before and after ticagrelor-intake. Uridine uptake was inhibited with 11%, but this did not reach statistical significance ( $P=0.056$ ). For the interpretation of these results, it is important to realize that after facilitated diffusion via the ENT, adenosine is rapidly metabolized, and that intracellular deamination of adenosine is rate limiting for adenosine uptake rather than ENT activity.<sup>25</sup> Only after pharmacological inhibition of >80% of ENT activity, the ENT transporter becomes rate limiting, and adenosine uptake is reduced.<sup>25</sup> As such, 11% ENT inhibition will not have any effect on extracellular adenosine concentration. Indeed, also in whole blood experiments, ticagrelor did not affect adenosine disappearance at relevant concentrations.

The results of our series of experiments contradict previous studies. *In vitro* Van Giezen et al reported in a canine model that ticagrelor augmented adenosine-induced vasodilation in the coronary artery.<sup>22</sup> However, this effect was observed only at a ticagrelor plasma concentration of 13.4  $\mu\text{M}$  and not with the lower concentration of 4.1  $\mu\text{M}$ , which is still considerably higher than the plasma concentration in patients treated with ticagrelor. In contrast, in another *in vitro* study, the addition of 1  $\mu\text{M}$  of ticagrelor to whole blood resulted in a slightly but significantly higher adenosine concentration 1 minute after the addition of 7.1  $\mu\text{M}$  of adenosine.<sup>26</sup> In healthy subjects *in vivo* Wittfeldt et al showed that a single dose of 180 mg of ticagrelor augmented the coronary blood flow velocity after intravenous adenosine infusion, which was prevented by concomitant administration of the adenosine receptor antagonist theophylline.<sup>27</sup> Similar results were obtained by Alexopoulos et al in patients with a recent non-ST-elevation acute coronary syndrome.<sup>28</sup> The route of administration in these studies is the major and important difference with our study: in our model adenosine is administered into the brachial artery, resulting in a high local arterial adenosine concentration, but without any systemic effects. Intravenous administration of adenosine, however, induces major systemic hemodynamic effects consisting of increased systolic blood pressure and heart rate and decrease of diastolic blood pressure by the combination of a direct vasodilator effect and a pronounced activation of sympathetic nervous system activity by stimulation of peripheral chemoreceptors.<sup>29</sup> Therefore, the coronary blood flow response in the Wittfeldt and Alexopoulos papers is driven by different effects, whereas adenosine-induced vasodilation in our study was not confounded by systemic hemodynamic and nervous effects. Finally, the

plasma concentration of ticagrelor in the study by Wittfeldt et al was slightly higher than in our study, despite the fact that in both studies the plasma concentration was determined 2 hours after intake of 180 mg ticagrelor in healthy subjects. Bonello et al measured plasma adenosine concentration in patients with an acute coronary syndrome who were randomized to either ticagrelor or clopidogrel at a time point of 6 hours after the loading dose.<sup>19</sup> They observed a significantly higher adenosine concentration in the patients treated with ticagrelor, and they observed a significant inhibition of *ex vivo* adenosine uptake in whole blood. It is likely that in the patients in this study, just as in the study by Alexopoulos et al, the adenosine metabolism is affected by the myocardial ischemia, the profound activation of the sympathetic nervous system by the coronary event, and by the comedication (e.g. statins that will increase the extracellular formation of adenosine by activation of the ecto-5'-nucleotidase<sup>12, 15</sup>). In addition, the pharmacological blocking solution that is used to completely block adenosine metabolism in the *ex vivo* whole blood experiments differs from the solution that we previously have validated to result in an almost complete recovery of adenosine.<sup>24</sup> The inconsistent results of our study and the Bonello study might be caused, at least in part, by these differences.

### Study limitations

Our study has several potential limitations. First, our experiments were performed in healthy subjects without cardiovascular disease and without concomitant cardiovascular drugs, such as in the study by Bonello et al.<sup>19</sup> Therefore, the results of our study do not exclude any effect of ticagrelor on the adenosine system in patients with acute coronary events. Secondly, we investigated adenosine formation and uptake in the forearm, in contrast to the coronary circulation that was studied by Wittfeldt et al.<sup>27</sup> However, ENT is not known to differ between heart and forearm and therefore we believe that extrapolation from forearm to heart is valid for this particular research question.

### Conclusions

Ticagrelor dose-dependently inhibits nucleoside transporter activity in isolated red blood cells of healthy volunteers. However, the plasma concentration of ticagrelor after normal dosing in humans is too low to result in a significant increase in extracellular adenosine and adenosine receptor stimulation via this mechanism. In addition, the lack of any effect of ticagrelor on dipyridamole-induced vasodilation suggests that ticagrelor does not augment endogenous extracellular adenosine formation. Our findings, therefore, argue against a role these mechanisms as an explanation for the effects of ticagrelor, including lower mortality, dyspnea, ventricular pauses, and modulation of inflammation, as observed in recent clinical studies.

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# 8

## General discussion and conclusions



Ischemic preconditioning and pharmacological preconditioning are powerful tools to reduce ischemia-reperfusion injury. Extensive research has been done in this field and promising results have been reported in different organs (e.g. the myocardium, brain and kidneys). Most of these results are based on animal experiments. In this thesis we have tried to translate these findings to the human *in vivo* setting. From the basic assumption that the adenosine pathway plays a crucial role in the cardioprotective effect of ischemic preconditioning, we investigated the effect of three different drugs that (might) target this pathway and that are already used in common clinical practise: metformin, dipyridamole and ticagrelor.

First, in an effort to develop a novel model of IR injury in humans, in **chapter 2** we explored whether strenuous exercise could be used to induce mild myocardial IR injury, detected by a rise in the plasma hs-troponin I concentration. We aimed to validate this model using remote ischemic preconditioning (RIPC). RIPC is a powerful strategy to limit IR-injury and therefore we expected RIPC to prevent post-exercise hs-troponin release. In a randomized controlled cross-over study in healthy volunteers, we studied the effect of RIPC on exercise-induced troponin I release. Although exercise indeed resulted in a temporary rise in troponin I, RIPC could not prevent or reduce this increase. Therefore, we argued that IR does not importantly contribute to this rise in troponin. Strenuous exercise, therefore, is not a valid model to study myocardial IR-injury.

In **chapter 3** we gave a more detailed introduction of the cardioprotective effects of the glucose lowering drug metformin. Large observational clinical trials reported that metformin has a positive effect on cardiovascular morbidity and mortality independent of glycemic control. In animal studies, metformin not only might affect cardiovascular risk factors for the development of atherosclerosis (e.g. lipid profile, bodyweight, blood pressure), but animal research has also revealed a direct cardio-protective effect via activation of the RISK pathway, AMPK and the adenosine pathway. Animal models (in both diabetic as well as non-diabetic animals) have provided consistent evidence that metformin can limit myocardial IR-injury. Based on these data, metformin would be an attractive candidate to limit myocardial IR injury in the clinical setting.

In **chapter 4** we performed the first human *in vivo* study investigating the effect of short-term pre-treatment with metformin on endothelial IR-injury. As a model we used flow mediated dilation of the brachial artery before and after prolonged ischemia and reperfusion of the forearm. The IR protocol significantly reduced FMD, but this was not prevented by metformin treatment.

The results of chapter 4, however, do not exclude a beneficial effect of metformin on myocardial IR-injury in the clinical setting for several reasons. First, the mechanism of endothelial IR-injury might differ from IR-injury in cardiomyocytes. In addition, our findings in the brachial artery might not be representative for the coronary circulation. Finally, the study presented in chapter 4 involved young healthy subjects, which differ substantially from the older patients with coronary artery disease. Therefore we readdressed our hypothesis in **chapter 5** in a prospective randomised clinical trial among non-diabetic patients undergoing elective coronary artery bypass surgery. We investigated whether metformin was able to reduce IR-injury associated with on pump heart surgery. We recruited 100 patients who were randomised between pre-treatment with metformin or placebo and measured post-operative hs-troponin I release as a measure of post-surgery myocardial damage. Secondary endpoints included the postoperative occurrence of arrhythmias, the need for inotropic support, time to detubation and ICU length of stay. Another secondary outcome was *ex-vivo* post-ischemic recovery of contractile function of isolated atrial trabeculae. In addition, we explored *ex-vivo* the activation of cardioprotective signaling pathways in isolated right atrial tissue. Our results showed that metformin did not limit IR-injury, detected with post-surgical plasma hs-troponin I release. Nor was there any effect on the secondary clinical endpoints. In accordance with these clinical endpoints, metformin did not improve the *ex vivo* post-ischemic recovery of contractile function in isolated atrial trabeculae. In our study we confirmed activation of AMPK and the RISK-pathway in atrial tissue, but to a smaller extent than in murine models. We concluded that the mild activation of AMPK and Akt by metformin was not sufficient to offer significant cardioprotection.

An alternative pharmacological agent to reduce IR-injury is dipyridamole. Dipyridamole augments the endogenous interstitial adenosine concentration by inhibiting the equilibrative nucleoside transporter (ENT). In preclinical animal studies, and studies in healthy volunteers and in patients undergoing coronary angioplasty (PCI), dipyridamole limits IR-injury.<sup>1-8</sup> In **chapter 6** we describe the results of a second randomised clinical trial that we have designed to test the hypothesis that oral pre-treatment with dipyridamole prevents IR-injury in patients who undergo elective CABG. 79 patients were included in the analysis that showed that dipyridamole did not limit myocardial IR-injury, as measured by post-operative hs-troponin I release. Also, there was no effect on the occurrence of post-operative arrhythmias, the need for inotropic support or the duration of ICU stay (secondary endpoints).

With adenosine as a key element in the modulation of IR-injury, the novel P2Y<sub>12</sub> receptor blocker ticagrelor presents a potential alternative strategy to influence myocardial damage caused by ischemia and reperfusion. In the PLatelet inhibition

And paTient Outcomes (PLATO) study ticagrelor reduced death from vascular causes, myocardial infarction or stroke compared to clopidogrel.<sup>9</sup> In the past years, evidence has accumulated that ticagrelor inhibits the cellular uptake of the endogenous nucleoside adenosine by blockade of the ENT and this mechanism has been proposed to contribute to the benefits of ticagrelor observed in the PLATO study.<sup>10</sup> Most of the evidence on the effect of ticagrelor on adenosine metabolism is based on *in vitro* studies, or from studies in patients in which the adenosine metabolism could be affected by other factors, such as co-medication. In **chapter 7** we aimed to test whether ticagrelor inhibits the ENT transporter in humans *in vivo* in relevant concentrations in a randomised placebo-controlled cross-over trial in 14 healthy male volunteers. We tested whether ticagrelor affects adenosine- and dipyridamole-induced forearm vasodilation measured by venous occlusion plethysmography. Adenosine- and dipyridamole-induced vasodilation is used as pharmacological tool to study adenosine uptake and extracellular adenosine formation, respectively. Change in forearm blood flow represent adenosine receptor activation. Finally, *ex vivo* uptake of adenosine and uridine in isolated red blood cells was measured. The main finding of our study was that a single dose of ticagrelor does not potentiate the forearm vasodilator response to adenosine and dipyridamole. In addition, there was no significant *ex vivo* nucleoside uptake inhibition observed in isolated red blood cells in these subjects after oral treatment with ticagrelor. These *in vivo* and *ex vivo* findings were consistent with additional *in vitro* studies in isolated red blood cells demonstrating that ticagrelor only inhibits nucleoside transport in concentrations that are considerably higher than those obtained after normal dosing. Altogether, our findings argue against a role for increased adenosine receptor stimulation as an explanation for the pleiotropic effects of ticagrelor

## Future perspectives and conclusions

Now that rapid reperfusion of the occluded artery in patients with an acute myocardial infarction is obtained in most patients, further efforts to improve tissue salvage after an ischemic event are based on limiting IR-injury. Animal data show profound benefit of (pharmacological) conditioning to limit IR-injury. Translation of these promising preclinical findings to the clinical setting appears to be notoriously difficult. There are many examples of this translation gap in the recent literature. Van den Worp et al. reported that in animal models of acute ischemic stroke, 500 'neuroprotective' strategies are effective in limiting infarct size, but only two of these are effective in humans.<sup>11</sup> With regard to metformin, preclinical studies report that metformin can reduce atherosclerosis, myocardial infarct size, and improve post-infarction remodeling in animal models. In the last two years, however, three randomized

clinical trials (including our MetCAB trial) demonstrated no relevant clinical benefit of metformin treatment on atherosclerosis, IR injury, nor post-infarction remodeling.<sup>12-14</sup>

The findings in this thesis are coherent with this phenomenon. There are several potential explanations for this discrepancy between preclinical and (our) clinical trial results. In general, failure of translation of promising preclinical findings to the clinical setting might be explained by methodological flaws in animal studies, e.g. lack of randomisation, lack of blinding, the lack of a formal sample size calculation and inadequate statistical analyses.<sup>15, 16</sup> Furthermore, translational failure can be caused by fundamental differences in biology and pathophysiology between laboratory animals and the patients for whom the treatment is intended. In addition, animal studies are predominantly performed in young and otherwise healthy animals. In contrast, cardiovascular patients are older, and have co-morbidities and co-medication, which can all interfere with cardioprotection.<sup>17</sup> An older age is associated with an impaired cardioprotective effect of preconditioning.<sup>17, 18</sup> Nowadays almost all cardiovascular patients, also the patients in our trials, use co-medication such as statins, nitrates and antiplatelet agents which offer cardioprotective effects themselves and limit potential additional value of novel agents.<sup>17, 19</sup> Finally, it is essential that the endpoint used in clinical trials resembles the preclinical experimental design. In the field of IR-injury, the vast majority of preclinical data concerns histological infarct size reduction with ischemic conditioning. Primary determinants of infarct size, such as area at risk and duration of the ischemic stimulus are well-controlled in the preclinical setting. Assessment of these relevant factors is more difficult in the clinical setting. In our thesis, we have used the setting of coronary artery bypass surgery as a model for the occurrence of ischemia reperfusion injury and we have determined post-operative hs-troponin I as a marker for IR-injury. The mechanism of troponin release in CABG might however differ from acute myocardial infarction. Where in acute myocardial infarction troponin release is inherently related to oxygen demand and supply mismatch, in CABG other mechanisms of injury such as direct trauma from surgical manipulation, intracoronary embolization and inflammation might affect plasma troponin concentrations.<sup>20</sup> Another complicating factor of the CABG-model, is that peri-operative use of anesthesia could also affect the results: propofol anesthesia can disrupt protection by ischemic preconditioning, while sufentanil and opioids have cardioprotective effects themselves thus may provide limited scope for added benefit. Indeed, although initial proof-of-concept studies on remote ischemic preconditioning in patients undergoing CABG showed promising results<sup>21, 22</sup>, more recent large multicenter RCT's could confirm relevant protective effects of RIPPC. Recently, the results of the *Effect of Remote Ischemic Preconditioning on Clinical Outcomes in Coronary Artery Bypass Grafting Surgery* (ERRICA) multicenter trial were published: not only the largest study up to now in the field of myocardial ischemia reperfusion

injury and the effect of RIPC in the setting of CABG, but also the first study to report on hard clinical endpoints.<sup>23</sup> The study included 1612 patients undergoing CABG surgery. Half of the patients were randomly selected to undergo RIPC induced by four 5-minute inflations and deflations of a standard blood pressure cuff while the other half underwent a sham procedure. Although the study did reveal a statistically significant reduction in post-operative hs-troponin by about 10% in the RIPC group, at 1 year there was no difference in the primary endpoint of MACCE (cardiovascular death, MI, stroke, and coronary revascularization) or any of its individual outcomes between the study and control groups. In the same issue of the *New England Journal of Medicine*, Meybohm et al reported that in 1403 patients undergoing CABG surgery, upper-limb RIPC performed while patients were under propofol-induced anesthesia did not show a relevant benefit among patients undergoing elective cardiac surgery.<sup>24</sup>

In combination with the above mentioned pitfalls of the CABG-model, these disappointing results of RIPC and pharmacological precondition on long-term clinical endpoints in the setting of CABG, seem to close the curtain for clinical application of preconditioning in this specific arena of cardiac surgery. In the clinical setting of an acute myocardial infarction, but also in the field of transplantation medicine, the concept of preconditioning, however, is still very promising. At this moment a number of large clinical trials are being conducted to further explore the effect of (pharmacological) conditioning on hard clinical endpoints in the setting of an ST-elevation myocardial infarction.<sup>25-27</sup> The results of these studies will determine future perspectives and value of preconditioning-based strategies to prevent reperfusion injury.



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# 9

## Samenvatting en discussie



Ischemische en farmacologische preconditionering zijn krachtige manieren om de schade die aan de hartspier ontstaat tijdens ischemie en het herstel van bloedtoevoer (ischemie-reperfusieschade), te verminderen. Verschillende onderzoeken op dit gebied (bijvoorbeeld in de hartspier, het brein en de nieren) hebben veelbelovende resultaten laten zien. De meeste van deze bevindingen zijn echter gebaseerd op dierexperimentele modellen. In dit proefschrift hebben wij geprobeerd deze bevindingen uit dierexperimentele modellen te vertalen naar de humane *in vivo* situatie. Het is bekend dat het beschermende effect van ischemische preconditionering voor een belangrijk deel loopt via adenosinereceptorstimulatie door endogeen adenosine. Daarom hebben wij in dit proefschrift drie geneesmiddelen onderzocht die een effect hebben op endogeen adenosine: metformine, dipyridamol en ticagrelor.

In **hoofdstuk 2** hebben wij een nieuw humaan model onderzocht voor het nabootsen van ischemie-reperfusieschade. Door mensen bloot te stellen aan intensieve fysieke inspanning werd geprobeerd milde myocardiale ischemie-reperfusieschade te induceren. Als maat voor deze schade werd de *high-sensitive* troponine I concentratie in het bloed gemeten. Ter validatie van dit model hebben wij gebruik gemaakt van *Remote Ischemic Preconditioning* (RIPC, ischemische preconditionering op afstand). RIPC is een bewezen effectieve manier om ischemie-reperfusieschade te verminderen en de verwachting was dan ook dat indien er ischemie-reperfusieschade op zou treden in dit model, RIPC deze schade zou verminderen dit zich zou vertalen in lagere *high-sensitive* troponine plasma spiegels na inspanning. In tegenstelling tot onze verwachting steeg het *high-sensitive* troponine I wel na intensieve inspanning, maar werd deze stijging niet beïnvloed door RIPC. Wij concludeerden dan ook dat dit protocol van intensieve inspanning geen goed model is om myocardiale ischemie-reperfusieschade te onderzoeken.

In **hoofdstuk 3** geven wij een gedetailleerd overzicht van de cardioprotectieve effecten van het glucoseverlagende medicijn metformine. Grote observationele studies hebben aangetoond dat metformine een gunstig effect heeft op de cardiovasculaire morbiditeit en mortaliteit van patiënten met diabetes mellitus type 2. Dit effect lijkt onafhankelijk van het effect van dit geneesmiddel op de glucoseregulatie. In dierexperimentele studies is aangetoond dat metformine niet enkel een invloed heeft op klassieke cardiovasculaire risicofactoren voor het ontwikkelen van atherosclerose (zoals bijvoorbeeld het lipidenprofiel, het lichaamsgewicht en de bloeddruk), maar dat er ook sprake is van een direct cardioprotectief effect via activatie van diverse cardioprotectieve signaaltransductieroutes, zoals de *RISK-pathway*, de AMPK- en de *adenosine-pathway*. Dierexperimentele studies (in zowel dieren met diabetes als zonder diabetes) hebben daarbij consistent aangetoond dat metformine myocardiale ischemie-reperfusieschade kan verminderen. Metformine is daarmee

een aantrekkelijke kandidaat om ook in patiënten myocardiale ischemie-reperfusieschade te verminderen.

In **hoofdstuk 4** beschrijven wij de resultaten van de eerste humane *in vivo* studie met metformine, waarbij het effect van kortdurende voorbehandeling met metformine op endotheliale ischemie-reperfusieschade is onderzocht bij gezonde vrijwilligers. Als model voor ischemie-reperfusieschade is gebruik gemaakt van *flow*-gemedieerde dilatatie (FMD) van de arteria brachialis. FMD is gemeten voorafgaand aan en na een periode van langdurige ischemie en reperfusie van de onderarm, waarbij de proefpersonen al dan niet waren voorbehandeld met metformine. Ischemie-reperfusie zorgde voor een significante afname van de FMD, maar deze afname werd niet voorkomen door voorbehandeling met metformine.

Dat wij in hoofdstuk 4 geen effect zagen van metformine op de endotheliale ischemie-reperfusieschade, hoeft niet meteen te betekenen dat er ook geen gunstige effecten van metformine te verwachten zijn op ischemie-reperfusieschade in het hart van patiënten. Het zou namelijk zo kunnen zijn dat het mechanisme van endotheliale ischemie-reperfusieschade anders is dan dat van myocardcellen. Verder zou het kunnen zijn dat onze bevindingen in de arteria brachialis niet representatief zijn voor de coronaire circulatie. Daarbij hebben wij onze studie zoals beschreven in hoofdstuk 4 uitgevoerd in jonge gezonde vrijwilligers, een studiepopulatie die op veel punten verschilt van de veelal oudere patiënten met coronaire hartziekte. Om deze redenen hebben wij in **hoofdstuk 5** onze hypothese opnieuw getoetst in een prospectieve gerandomiseerde klinische trial, uitgevoerd in patiënten (zonder diabetes mellitus) die een electieve *coronary artery bypass grafting* (CABG; bypassoperatie) zouden ondergaan. Wij onderzochten bij deze patiënten het effect van voorbehandeling met metformine op ischemie-reperfusieschade die optreedt tijdens de uitvoer van *on-pump* open hartoperatie. Wij includeerden 100 patiënten en randomiseerden deze naar voorbehandeling met metformine of met placebo. Als maat voor ischemie-reperfusieschade hebben wij hs-troponine I concentraties gemeten op verschillende tijdstippen na het verwijderen van de klem op de aorta. Secundaire eindpunten waren het optreden van ritmestoornissen na de operatie, de noodzaak voor inotropische ondersteuning, tijd tot detubatie en duur van de opname op de *intensive care*. Bij alle patiënten werd tevens een klein stukje atriaal weefsel weggehaald tijdens de operatie dat wij in het laboratorium hebben onderzocht. Dit weefsel hebben we gebruikt om *ex-vivo* post-ischemisch herstel van de contractiliteit van geïsoleerde atriale trabeculae te meten. Daarnaast hebben wij *ex-vivo* ook de activatie van verschillende cardio-protectieve signaaltransductieroutes in geïsoleerde trabeculae onderzocht. Onze resultaten toonden aan dat metformine geen effect had op de postoperatieve hs-troponineconcentratie in plasma, noch op de klinische secundaire uitkomstmaten.

In lijn met deze bevindingen, werd er ook geen effect van metformine op het *ex-vivo* post-ischemische herstel van de contractiliteit van geïsoleerde atriale trabeculae gevonden. In onze analyse zagen wij wel dat er sprake was van activatie van AMPK en van de *RISK-pathway* in atriaal weefsel, maar in een veel mindere mate dan in dierexperimentele modellen is beschreven. Wij concluderen dat de milde activatie van AMPK en Akt door metformine niet voldoende is geweest om te leiden tot (klinisch) relevante cardioprotectie.

Een andere mogelijkheid om farmacologisch ischemie-reperfusieschade te kunnen reduceren is het middel dipyridamol. Dipyridamol verhoogt de endogene interstitiële adenosineconcentratie door de *equilibrative nucleoside transporter* (ENT) te remmen. In preklinische dierstudies, studies bij gezonde vrijwilligers en bij patiënten die een percutane coronaire angioplastiek (PCI) ondergaan, is aangetoond dat dipyridamol ischemie-reperfusieschade vermindert.<sup>1-8</sup> In **hoofdstuk 6** beschrijven wij de resultaten van een tweede gerandomiseerde klinische trial bij patiënten die een electieve CABG ondergaan die wij hebben ontworpen om te onderzoeken of voorbehandeling met dipyridamol ischemie-reperfusieschade kan verminderen. In deze studie werden 79 patiënten geïnccludeerd en gerandomiseerd naar voorbehandeling met dipyridamol of placebo. Ook dipyridamol had bij deze patiënten geen effect op de myocardiale ischemie-reperfusieschade (gekwantificeerd door middel van postoperatieve hs-troponine I concentraties). Ook was er geen effect op het voorkomen van post-operatieve ritmestoornissen, de noodzaak voor inotropie of de duur van opname op de *intensive care* (secundaire eindpunten).

Uitgaande van de centrale rol die adenosine speelt in de modulatie van ischemie reperfusieschade, biedt de (relatief nieuwe) P2Y<sub>12</sub> receptorblokker ticagrelor potentieel nieuwe mogelijkheden om myocardiale ischemie reperfusieschade te beïnvloeden. De *PLatelet inhibition And paTient Outcomes* (PLATO) studie toont dat behandeling met ticagrelor in vergelijking met clopidogrel een reductie geeft van cardiovasculaire mortaliteit, het optreden van myocardinfarcten of ischemische cerebrovasculaire accidenten bij patiënten met een acuut coronair syndroom.<sup>9</sup> Dit effect was groter dan redelijkerwijs alleen kon worden toegewezen aan het plaatjesremmende effect. De afgelopen jaren is er steeds meer bewijs gekomen dat ticagrelor mogelijk ook de cellulaire opname van endogeen adenosine vermindert door inhibitie van de ENT, waardoor er meer extracellulair adenosine beschikbaar is om te binden aan de adenosinereceptor. Dit fenomeen wordt nu aangedragen als potentiële verklaring van de extra gunstige effecten van ticagrelor in de PLATO studie.<sup>10</sup> Het bewijs dat ticagrelor een effect heeft op het metabolisme van adenosine is grotendeels gebaseerd op *in vitro* studies of op studies in patiënten waarbij het adenosinemetabolisme ook beïnvloed zou kunnen zijn door andere factoren, zoals co-medicatie.



In **hoofdstuk 7** hebben wij onderzocht of ticagrelor daadwerkelijk de ENT transporter blokkeert in 14 mannelijke gezonde vrijwilligers, in een gerandomiseerde placebo gecontroleerde *cross-over* studie. Wij hebben daarbij onderzocht of ticagrelor een effect heeft op adenosine- en dipyridamol-geïnduceerde vasodilatatie in de onderarm, gemeten met behulp van veneuze occlusie plethysmografie. Wij hebben adenosine- en dipyridamol-geïnduceerde vasodilatatie als parameters gebruikt om respectievelijk cellulaire adenosine-opname en extracellulaire adenosinevorming te bestuderen. Daarbij is de mate van toename van de onderarmsdoorbloeding een maat voor activatie van de adenosinereceptor. Wij hebben ook *ex vivo* de opname van adenosine en uridine gemeten in geïsoleerde rode bloedcellen. De hoofdbevinding van onze studie was dat een enkele gift ticagrelor niet leidt tot een toename van de vasodilatatie van de onderarmsbloedvaten in respons op adenosine en dipyridamol. Ook leidt voorbehandeling met ticagrelor niet tot een significante inhibitie van opname van adenosine in geïsoleerde rode bloedcellen. Deze *in vivo* en *ex vivo* bevindingen waren in overeenstemming met aanvullende *in vitro* onderzoeken in geïsoleerde rode bloedcellen die aantoonde dat ticagrelor enkel de nucleosidetransporter remt in concentraties die veel hoger zijn dan de concentraties die verkregen worden tijdens normaal klinisch gebruik bij patiënten. Al met al pleiten onze bevindingen tegen de hypothese dat de pleiotrope effecten van ticagrelor in de klinische setting verklaard kunnen worden door een toename in adenosinereceptorstimulatie.

## Toekomstperspectieven en conclusies

Op dit moment bestaat de behandeling van patiënten met een acuut myocardinfarct uit het zo snel mogelijk openen van de afgesloten kransslagader op de catheterisatiekamer. Ondanks deze behandeling is de mortaliteit en morbiditeit (het optreden van hartfalen) aanzienlijk. De schade die ontstaat ten gevolge van het openen van het vat (ischemie-reperfusieschade) speelt daar een rol in. Het is daarom belangrijk om te proberen de ischemie-reperfusieschade van het myocard verder te verminderen. Dierexperimentele studies toonden duidelijk hoopvolle effecten van (farmacologische) preconditionering. De resultaten van deze veelbelovende preklinische studies laten zich echter niet zo makkelijk vertalen naar de klinische (humane) setting. Deze 'translatiekloof' is niet ongevoel in de literatuur. Zo beschreef Van den Worp *et al* dat in dierexperimentele modellen van een acuut herseninfarct zich in totaal 500 'neuroprotectieve' strategieën bewezen hebben in het effectief reduceren van infarctgrootte, maar dat slechts 2 van deze strategieën uiteindelijk effectief zijn gebleken in de klinische humane setting.<sup>11</sup>

Ook wij zien een soortgelijk fenomeen. Ten aanzien van metformine zijn er veel preklinische studies die aantonen dat metformine een gunstig effect heeft op atherosclerose, op hartinfarctgrootte en op post-infarct *remodeling*. Recent daarentegen zijn er drie gerandomiseerde klinische studies verricht (inclusief onze eigen MetCAB studie), die geen gunstig effect van metformine laten zien op atherosclerose, ischemie-reperfusieschade of op postinfarct *remodeling* in patiënten.<sup>12-14</sup>

Ook in dit proefschrift is het herhaaldelijk niet gelukt om veelbelovende preklinische bevindingen te vertalen naar de klinische praktijk. Er zijn verschillende mogelijke verklaringen voor deze discrepantie tussen preklinische bevindingen en (onze) klinische resultaten. Een eerste mogelijke verklaring betreft een probleem met de interne validiteit: namelijk de methodologische beperkingen van dierstudies. Zo is er in dierstudies vaak geen sprake van randomisatie tussen de verschillende behandelarmen, is er vaak geen blinding van de onderzoeker voor de behandeling, worden er zelden *sample size* calculaties uitgevoerd en kan er sprake zijn van onjuiste statistische analyses.<sup>15, 16</sup> Een andere verklaring betreft een beperkte externe validiteit: het falen van vertalen van bevindingen vanuit de preklinische studies naar de klinische praktijk kan ook verklaard worden door fundamentele verschillen in biologie en pathofysiologie tussen (laboratorium)dieren en de patiënten voor wie de therapie uiteindelijk bedoeld is. Daarbij is het zo dat dierexperimentele studies veelal worden uitgevoerd in jonge, gezonde dieren. Patiënten met hart- en vaatziekten daarentegen zijn veelal ouder en hebben vaak ook veel co-morbiditeit en gebruiken diverse medicijnen. Al deze factoren kunnen invloed kunnen hebben op de effectiviteit van cardioprotectieve interventies.<sup>17</sup> Oudere leeftijd is geassocieerd met een verminderd cardioprotectief effect van preconditionering.<sup>17, 18</sup> Tegenwoordig gebruiken vrijwel alle patiënten met hart- en vaatziekten, ook de patiënten in onze studies, medicatie, zoals statines en trombocytenaggregatieremmers die zelf ook al kunnen beschermen tegen ischemie-reperfusieschade en zo potentieel minder ruimte laten voor additionele bescherming door ischemische of farmacologische preconditionering.<sup>17, 19</sup> Ten slotte is het essentieel dat de eindpunten die gebruikt worden in de klinische trials ook overeenkomen met het design van de preklinische experimenten. Op het gebied van ischemie-reperfusieschade betreft het in het merendeel van de dierstudies reductie van histologisch bepaalde infarctgrootte door middel van ischemische preconditioning. Eindpunten zoals infarctgrootte, *area at risk* en duur van de ischemische stimulus zijn uitermate gecontroleerd in de preklinische experimenten. In de klinische praktijk daarentegen is het meten van dit soort eindpunten zeer moeilijk of niet mogelijk. In dit proefschrift is het ondergaan van een electieve CABG (hartoperatie) als model gesteld voor het optreden van ischemie-reperfusieschade. Als maat voor de opgetreden ischemie-reperfusieschade en daarmee primair eindpunt, is gekozen voor de plasmaconcentratie (verandering) van hs-troponine. De vraag rijst of het

mechanisme dat ten grondslag ligt aan de troponine-afgifte rondom een CABG gelijk is aan de situatie tijdens een acuut hartinfarct. Tijdens het acute hartinfarct is de (verhoogde) afgifte van hs-troponine (enkel) het gevolg van celverval door een *mismatch* tussen zuurstofbehoefte en aanbod. Rond een CABG kunnen ook andere mechanismes een rol spelen, zoals schade veroorzaakt door chirurgische manipulatie, het optreden van intracoronaire embolisatie en het ontstaan van een ontstekingsreactie. Al deze elementen kunnen een direct effect hebben op de afgifte van troponine.<sup>20</sup> Een andere complicerende factor van het CABG model is dat er rondom een dergelijke operatie gebruik wordt gemaakt van diverse anesthetica, middelen die op hun beurt ook een effect kunnen hebben op de resultaten: propofol kan het protectieve effect van preconditionering tegengaan, terwijl sufentanyl en opiaten zelf ook een cardioprotectief effect kunnen hebben, waardoor er wellicht weinig ruimte overblijft voor een additioneel gunstig effect van de door ons gebruikte medicamenten. In het verleden hebben verschillende studies met ischemische preconditionering op afstand bij patiënten die een CABG ondergingen veelbelovende resultaten laten zien.<sup>21, 22</sup> Recent zijn de resultaten van de '*Effect of Remote Ischemic Preconditioning on Clinical Outcomes in Coronary Artery Bypass Grafting surgery*' (ERRICA) multicenter trial gepubliceerd: niet alleen de grootste studie tot nu toe op het gebied van RIPC bij patiënten die een CABG ondergaan, maar ook de eerste studie die effect van RIPC onderzocht opharde klinische eindpunten.<sup>23</sup> In deze studie zijn in totaal 1612 patiënten geïncludeerd die een CABG zouden ondergaan. De helft van de patiënten werd gerandomiseerd naar RIPC (vier episodes van 5 minuten van ischemie van de onderarm door oppompen van een bloeddrukmanchet) terwijl de andere helft een *sham* (nep) procedure onderging. Hoewel de studie een statistisch significante reductie in post-operatieve hs-troponine van ongeveer 10% aantoonde in de RIPC groep, was er na 1 jaar geen verschil tussen de twee groepen wat betreft het primaire eindpunt: een combinatie van cardiovasculaire sterfte, het optreden van een myocardinfaarct, beroerte of coronaire revascularisatie of elk van deze eindpunten afzonderlijk. In dezelfde uitgave van de *New England Journal of Medicine* rapporteerden Meybohm *et al* hun studieresultaten in 1403 patiënten die een CABG ondergingen, waarbij RIPC van de bovenarm werd uitgevoerd terwijl patiënten onder anesthesie waren met propofol: ook in deze studie waren er geen verschillen in vergelijking met patiënten die deze voorbehandeling niet kregen.<sup>24</sup>

Door de teleurstellende uitkomsten van onderzoeken naar het effect van RIPC en farmacologische preconditionering op de relevante klinische eindpunten, lijkt het doek te vallen voor preconditionering als strategie om ischemie-reperfusieschade te verminderen in de setting van CABG operaties. In de setting van een acuut myocardinfaarct daarentegen, maar ook op het gebied van de transplantatiegeneeskunde, is het concept van preconditionering nog steeds veelbelovend. Op dit moment is er

een aantal grote klinische trials bezig om het effect van (farmacologische) preconditionering op harde klinische eindpunten te onderzoeken bij patiënten met een ST-elevatie myocardinfarct.<sup>25-27</sup> De resultaten van deze studies zullen de toekomstperspectieven en de waarde van preconditioneringsstrategieën om ischemie-reperfusieschade te voorkomen verder bepalen.

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Dankwoord

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## Dankwoord

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Saloua El Messaoudi werd op 26 december 1982 geboren in Arnhem. Na het behalen van haar eindexamen van het Atheneum B aan het Thomas à Kempis College te Arnhem, startte zij in 2002 met haar Geneeskunde opleiding aan het Radboudumc te Nijmegen. Al tijdens haar geneeskundestudie raakte zij geïnteresseerd in wetenschappelijk onderzoek, hetgeen in 2006 resulteerde in haar eerste publicatie in het Nederlands Tijdschrift voor de Geneeskunde. Ook tijdens haar wetenschappelijke stage heeft zij zich verdienstelijk gemaakt voor de wetenschap en onderzoek gedaan naar het effect van vitamine C op het sympatisch zenuwstelsel. Al vroeg in haar opleiding bleek er een sterke voorliefde te zijn voor de Cardiologie. Na het afronden van haar geneeskundestudie, werd zij na een korte periode in het Radboudumc te hebben gewerkt, in juli 2008 door prof. dr. F.W.A. Verheugt voor de opleiding tot cardioloog aangenomen. Aangezien haar ambities verder strekten dan alleen het afronden van haar specialisme, werd al snel besloten de opleiding te combineren met een promotietraject. Onder leiding en toezicht van prof dr. G. Rongen en prof. Dr. N.P. Riksen werd een subsidie van de hartstichting verworven die haar in staat stelde haar promotietraject aan te vangen. Het feit dat het onderwerp raakvlakken had met de Cardiologie, Vasculaire Geneeskunde en Cardio-thoracale Chirurgie, gaf haar de mogelijkheid als verbinding tussen deze specialismen op te treden en zij onderhoudt nog steeds warme banden met de verschillende groepen. Haar vooropleiding Interne Geneeskunde volgde zij in het Canisius Wilhelmina Ziekenhuis te Nijmegen (opleider: dr. T. Dofferhof) en zij maakt nog dagelijks gebruik van de kennis die zij daar heeft opgedaan. Haar B-jaar cardiologie volgde zij met veel plezier aan de afdeling Cardiologie van het Rijnstate Ziekenhuis te Arnhem (opleider: dr. H. Bosker) en haar ervaringen met deze opleidingsgroep hebben de belangrijke basis gelegd voor de cardioloog die zij in de toekomst hoopt te worden. Sinds 2015 is zij als AIOS Cardiologie weer werkzaam op de afdeling Cardiologie van het Radboudumc en op 1 juli 2017 zal zij haar opleiding tot cardioloog afronden. Tijdens haar opleiding heeft zij altijd geprobeerd zich zo breed mogelijk te ontwikkelen, zo is zij o.a. lid van het dagelijks bestuur van de Centrale Opleidingscommissie en is zij actief binnen de AAVR. Daarnaast heeft onderwijs haar passie en zij heeft o.a. gewerkt als gastdocent op de Hoge School Arnhem Nijmegen.





## List of publications

1. Gomes ME, **El Messaoudi** S, Lenders JW, Bellersen L, Verheugt FW, Smits P, Tack CJ. High dose ascorbic acid does not reverse central sympathetic over-activity in chronic heart failure. *J Clin Pharm Ther.* 2011;36(5):546-52
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